PB-213 006

INORGANIC SULFUR OXIDATION BY IRON-OXIDIZING BACTERIA

Donald G. Lundgren

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Syracuse, New York

June 1971

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SELECTED WATER RESOURCES ABSTRACTS

INPUT TRANSACTION FORM

1. Report No.

2.

3. Accession No. **W** 73-01609

4. Title

INORGANIC SULFUR OXIDATION BY IRON-OXIDIZING BACTERIA,

5. Report Date

6.

8. Performing Organization Report No.

7. Author(s)

Lundgren, D~~onald~~ G.

10. Project No.

EPA Program 14010 DAY 06/71

9. Organization

Syracuse Univ., New York. Dept. of Biology

11. Contract/Grant No.

13. Type of Report and Period Covered

12. Sponsoring Organization

EP2.10:14010 DAY 06/71

15. Supplementary Notes

Environmental Protection Agency, Water Pollution Control Research Series, ~~Program 14010 DAY~~, June 1971. 149 p. 21 fig., 32 tab., 98 ref.

16. Abstract

Sulfur metabolism of Thiobacillus ferrooxidans and rhodanese-sulfite oxidase enzymes were studied. As disclosed by electron microscope examination, the purified cell envelope of lipopolysaccharide contains a very high quantity of ferric iron. A new colorimetric whole cell assay was developed to study iron oxidation. The presence of pyrophosphatase enzyme was revealed for the first time in chemolithotrophic microorganisms. Study of energy sources disclosed that the chemolithtrophic T. ferrooxidans can evolve heterotrophic mechanisms and grow on glucose, following the Entner-Doudoroff pathway. (Wilde-Wisconsin)

17a. Descriptors

*Mine drainage, *Biochemistry, *Iron bacteria, *Thiobacillus ferrooxidans, *Analytical techniques, Oxidation, Sulfur, Iron, Colorimetry, Enzymes, Metabolism, Hydrogen ion concentration, Acid mine water, Cytological studies

17b. Identifiers Glucose metabolism

Copy available from GPO Sup Doc
, $1.25 ; microfiche
from NTIS as PB 213006, $0.95.

17c. COWRR Field & Group 05C, 05B, 05A

18. Availability

19. Security Class. (Report)

20. Security Class. (Page)

21. No. of Pages

22. Price

Send To:

WATER RESOURCES SCIENTIFIC INFORMATION CENTER
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D. C. 20240

Abstractor S. A. Wilde va

Institution -1a- Wisconsin Univ., Madison

Inorganic Sulfur Oxidation by Iron-Oxidizing Bacteria

by

Department of Biology
Syracuse University
Syracuse, New York

Details of illustrations in
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studied on microfiche.

for the

ENVIRONMENTAL PROTECTION AGENCY

Project #14010 DAY

June, 1971

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Stock Number 5501-0117

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Abstract

The utilization of sulfur and reduced sulfur compounds by the iron-oxidizing chemolithotroph Thiobacillus ferrooxidans was studied at the biochemical level. The identification, characterization and partial purification of the rhodanese and sulfite oxidase enzymes completed the scheme of sulfur metabolism in T. ferrooxidans which leads to energy generation.

The cell envelope lipopolysaccharide (LPS) purified from iron-grown cells was studied in the electron microscope. The partial chemical composition of the LPS revealed unusually high quantities of Fe^{3+}. A new colorimetric whole cell assay to study iron-oxidation kinetics was developed which will be of benefit to future studies at the molecular level. The inorganic pyrophosphatase enzyme, an essential enzyme in maintaining the energy balance in the cell, was partially purified and its properties studies. This is the first account of the presence of this enzyme in chemolithotrophic microorganisms.

The effects of organic carbon and energy sources on chemolithotrophic microorganisms were studied. T. ferrooxidans can convert from chemolithotrophic to heterotrophic metabolism after a long lag in the presence of the organic substrate, and after some energy is stored from iron oxidation. Growth on glucose proceeds much like other heterotrophic gram negative organisms. The metabolism of glucose is via the Entner-Doudoroff pathway.

A new species of Thiobacillus has been isolated from alkaline mine drainage ($pH > 4.5$) and its taxonomic status determined. The organisms most interesting feature is its ability to produce basic substances which can increase the pH to as much as 8.3.

This report was submitted in fulfillment of grant number 14010 DAY under the sponsorship of the Federal Water Quality Administration.

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SECTION I

CONCLUSIONS

The studies presented herein describe basic laboratory research on the structure and function of *T. ferrooxidans* grown under different conditions relating to acid mine drainage. The purpose of these basic studies is to try to find a specific aspect of the structure or metabolism of *T. ferrooxidans* which can be used as a means of abating acid pollution at its source. Specific conclusions in this regard are as follows:

(1) The inhibition of the rhodanese enzyme by iodoacetamide and N-ethyl-maleimide indicate that thiol groups are required for activity.

(2) The inhibition of the sulfite oxidase enzyme by low (40 μM) concentrations of phosphate would be of practical use.

(3) The absolute requirement of Mg^{++} for inorganic pyrophosphatase activity.

(4) The inhibition of inorganic pyrophosphatase activity by ethylenediaminetetraacetate.

(5) The cell envelope lipopolysaccharide is important in maintaining a balance between the environmental pH and the intracellular pH.

(6) When a heterotrophic substrate such as glucose is included in the medium, *T. ferrooxidans* will convert from chemolithotrophic to heterotrophic growth after a period of energy storage.

(7) Growth of *T. ferrooxidans* on glucose as the sole carbon and energy source does not result in the generation of acid, sulfate or ferric precipitates.

(8) The isolation and identification of a *Thiobacillus* species which produces alkaline products.

(9) More basic research needs to be done on *T. ferrooxidans*.

SECTION II

RECOMMENDATIONS

The following recommendations are made based on the aforementioned conclusions of this research:

(1) More basic research into the function of the cell envelope as it relates to the oxidation of a soluble inorganic substrate, like iron; the oxidation of an insoluble inorganic substrate, like sulfur; the oxidation of an organic substrate like glucose.

(2) Basic research into how the cell envelope maintains a balance in pH between the acidic environment and the less acid cytoplasm. It appears that the outer cell envelope will be the site of the cell which will be ameanible to the action of inhibitor compounds in the acid mine drainage environment.

(3) More research must be done on the effects of chemical inhibitors on the key enzymes involved in iron and sulfur utilization. Preferably, the inhibitors would be specific enough to not cause other pollution problems.

(4) More research in the field, relating to trying to adapt the iron and sulfur utilization to heterotrophic growth. This is a logical step since heterotrophic growth does not lead to the generation of acid, sulfate or ferric precipitates.

(5) More studies into the isolation and identification of microbes which will tolerate and grow in acid mine drainage, but will generate alkaline byproducts. This would help to raise the pH of mine drainage as well as causing less ferric precipitate to be formed. Such an organism could successfully compete with *T. ferrooxidans* and render it ineffective since it is obligately acidophilic.

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SECTION III

INTRODUCTION

The iron oxidizing bacteria of the genus Thiobacillus are widely distributed in acid waters associated with deposits of metal sulfides and sulfide bearing coals. They are indigenous to environments containing metal sulfides, oxygen (either atmospheric or in aqueous solution) and acid conditions. The iron sulfides, mascarite and pyrite, are found associated with coal strata and upon removal of the coal, the sulfur bearing iron compounds are exposed to air and water. This exposure establishes the first step in developing an ecosystem which will maintain sulfur and iron oxidizing bacteria. Both chemical and biological oxidation of the iron containing minerals lead to sulfuric acid and the red to red-brown sludge deposits of oxidized iron precipitates which are associated with acid mine drainage. The generation of the sulfuric acid and ferric precipitates, called "yellow boy", are a major water pollution problem in this country and elsewhere. It has been estimated that 10,000 miles of streams and 29,000 surface acres of impoundments and reservoirs are seriously affected by acid mine drainage. The drainage from active and abandoned mines amount to over 4 million tons of acidity per year in this country.

The magnitude of this pollution problem and its concomitant economic and environmental problems have prompted the study of the causes of this type of pollution, with hopes that some aspect of the generation of acid mine drainage may be found that can be exploited to stop the causative reaction.

The basic reactions involved in the generation of insoluble ferric precipitates and acid can be expressed chemically as follows:

$$\underset{\text{(pyrite)}}{2FeS_2} + 2H_2O + 7O_2 \longrightarrow \underset{\text{(ferrous sulfate)}}{2FeSO_4 + 2H_2SO_4} \qquad (1)$$

$$4FeSO_4 + 2O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (2)$$

$$Fe_2(SO_4)_3 + 6H_2O \longrightarrow \underset{\text{(ferric hydroxide)}}{2Fe(OH)_3 + 3H_2SO_4} \qquad (3)$$

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Pyrite oxidation can also occur in the presence of ferric iron

$$FeS_2 + 14Fe^{3+} + 8H_2O \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad (4)$$

This reaction (4) also generates acid thus keeping the pH low so that the reaction may occur in acid streams. However, reaction (2) proceeds slowly below pH 4 in the absence of a catalyst, but proceeds rapidly in the presence of iron oxidizing bacteria. Thus one might consider that the regulation of the production of acid mine drainage conditions depends upon the rate of reaction (2) which has been found to be controlled by the presence of iron oxidizing bacteria (Thiobacillus ferrooxidans). The bacteria have been shown to accelerate reaction (2) by a factor of more than 10^6 over the chemical reaction rate (1). Thus the generation of Fe^{3+} in reaction (2) can cause the chemical oxidation of pyrite (reaction (4)) producing more acid.

Thiobacillus ferrooxidans can also oxidize sulfur and sulfur containing compounds to sulfuric acid. Understanding the metabolism of these sulfur compounds is also important to the study of methods for control of acid mine drainage. In the biogeochemical sulfur cycle in nature, the sulfur oxidizing bacteria play an important oxidative role by converting the reduced sulfur compounds (H_2S and S°) to oxidized sulfur compounds (SO_3^{2-}, $S_2O_3^{2-}$, SO_4^{2-}, etc). The anaerobic sulfate reducing bacteria complete the cycle by reducing the oxidized sulfur compounds back to the reduced state.

When one refers to iron oxidizing bacteria or sulfur oxidizing bacteria, one is describing the mode of energy generation for the bacterium, i.e. by oxidation of reduced iron and sulfur compounds. These types of bacteria obtain their carbon for cell growth and reproduction from CO_2, thus the organisms are referred to as chemolithotrophs, or simply, autotrophs. In acid mine drainages, there are small but significant quantities of organic carbon which could support heterotrophic growth, that is, growth and energy production are derived from the metabolism of organic compounds. Thus, it would be important to study the metabolism of organic compounds by iron and sulfur oxidizing bacteria to try to determine if there is some way in which to "turn off" the oxidations of the iron and sulfur compounds which produce acid and ferric precipitates, and "turn on" oxidation of organic compounds which would not produce acid or ferric precipitates.

The research performed under this grant involved four general areas related to the basic study of the microbe which causes acid mine pollution. Because of this, the body of the report will be divided into four separate sections, and each section will have its own description of materials and methods used, results obtained and the significance of the results found. The four general areas involve studies of sulfur metabolism, the isolation and characterization of new thiobacillus species from alkaline mine drainage, iron metabolism, and heterotrophic growth of *T. ferrooxidans* under acid conditions.

SECTION IV

STUDIES OF THE METABOLISM OF THIOBACILLUS FERROOXIDANS GROWN ON ELEMENTAL SULFUR

Introduction

Thiobacillus ferrooxidans, formerly called Ferrobacillus ferrooxidans can use elemental sulfur as the sole source of energy, oxidizing the sulfur to sulfate. The oxidation is via sulfite involving the sulfur oxidizing enzyme (2). Thiosulfate can be formed by a chemical condensation of sulfite and sulfur. This thiosulfate has also been found to be converted to tetrathionate by the thiosulfate oxidizing enzyme (3). Thiosulfate can be converted to sulfur and sulfite by the enzyme rhodanese, first discovered by Sorbo (4) in 1953 in ox liver. Sulfite can be oxidized to sulfate by sulfite oxidase, with the concomitant production of ATP energy for cell growth. Both of these latter enzymes have been studied in other Thiobacillus species (5, 6, 7, 8, 9, 10, 11, 12) but their presence in T. ferrooxidans was not determined until the present work. The methods used and results obtained for each enzyme isolation will be presented separately, followed by a discussion of both enzymes.

Isolation and Properties of the Rhodanese Enzyme.

Materials and Methods

Organism

Thiobacillus ferrooxidans (Ferrobacillus ferrooxidans) was grown on the 9K salts-elemental sulfur medium, harvested after 7 days growth, and washed as previously reported (13).

Preparation of Cell-Free Extracts and Enzyme Purification

Cells were treated with 0.5 M Tris-HCl buffer (pH 8.5) overnight at 4°C, and then centrifuged and resuspended in 0.01 M Tris-HCl containing 10^{-3}M $Na_2S_2O_3$ at pH 7.8 (Buffer A) to form a 20% (w/v) cell suspension; thiosulfate was added to help stabilize the enzyme (5). The cells were disrupted in a water-cooled (6-8°C),

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10 kcycles/s Raytheon sonic oscillator for 20 min. Whole cells and debris were removed by centrifugation at 13,000 x g for 20 min. The supernatant, containing the crude extract, was then centrifuged at 105,000 x g for 1 hr at 4°C in the Beckman model L-2 ultracentrifuge equipped with a type 65 angular rotor. The clear, yellow supernatant from this step was removed, and the pellet resuspended in buffer A and recentrifuged at 105,000 x g for 1 h. The two supernatants (containing the enzyme) were combined. About 99% of the enzymatic activity of the crude cell-free extract was found in the supernatant fraction.

Supernatant fraction (27 ml) was treated with cold 1 N acetic acid to a pH of 5.0, and a volume, equal to the supernatant fraction, of 2% streptomycin sulfate was added; the resulting turbid solution was stirred for 30 min in an ice bath at 0-4°C. Precipitated protein and nucleic acids were removed by centrifugation and discarded. To the resulting supernatant, solid ammonium sulfate was slowly added to 25% saturation (while maintaining the pH at 5.0 with 1 N acetic acid) and stirred for 30 min in an ice bath. After centrifuging and discarding the pellet, the supernatant was treated with ammonium sulfate to 90% saturation, stirred for 30 min, and allowed to stand for at least 1 h in an ice bath. The precipitate was recovered by centrifugation, resuspended to the original volume with buffer A, and dialyzed overnight against 21 liters of the same buffer. The dialysis buffer was then changed and the preparation dialyzed for an additional 3 h. To the dialyzed extract, and equal volume of a 20% slurry of DEAE-cellulose in buffer A was added and stirred for 1 h at 4°C. The enzyme, adsorbed to the cellulose, was recovered after centrifugation and elution with 30 ml each of buffer A of 0.05, 0.1, and 0.25 M. Enzyme of highest specific activity was eluted at 0.25 M buffer. Further washing of the cellulose at higher concentrations of buffer removed little additional enzyme.

Protein was estimated according to the procedure of Lowry et al. (16) with crystalline bovine serum albumin used as the standard.

Enzyme Assays

Rhodanese was assayed using a modification of the procedure of Bowen et al. (5) with thiocyanate measured colorimetrically. The reaction mixture, unless otherwise indicated, contained 500 µmoles of Tris-HCl buffer (pH 8.5), 50 µmoles of $Na_2S_2O_3$, 50 µmoles of NaCN (prepared in 0.1 M Tris-HCl, pH 8.5), enzyme (usually 0.1 ml), and water to a total volume of 5 ml. The reaction was initiated with the addition of NaCN to individual screw-cap reaction tubes, and the reaction proceeded at room temperature. At the desired time, 0.1 ml of 38% formaldehyde was added to stop the reaction and 0.5 ml of the ferric nitrate reagent of Sorbo (20% $Fe(NO_3)_3$ in 3 N NHO_3) was added. The reaction tubes were then centrifuged to remove denatured protein and the intensity of the orange-yellow color was measured colorimetrically in a Klett-Summerson photoelectric colorimeter, using a blue (No. 42) filter. Thiocyanate levels were determined by comparison to known standards; reagent blanks were prepared by reversing the order of the addition of cyanide and formaldehyde. Either the 90% ammonium sulfate fraction or the DEAE-cellulose eluate was used in all enzyme assays.

Chemicals

All reagents were obtained from commercial sources; enzyme grade ammonium sulfate from Nutritional Biochemicals, sodium thiosulfate from J. T. Baker Chemical Company, sodium cyanide and ferric nitrate from Fisher Scientific Company, Tris from Sigma Chemical Company, and DEAE-cellulose from Schleicher and Schull Inc. All reagents were prepared in glass-distilled water.

Results

Purification of the Enzyme

Table 1 lists the enzyme purification steps and shows a 38-fold purification. All fractions were stable at room temperature for approximately 4 h. After 24 h at 4°C enzyme activity was gradually lost, which probably explains the different specific activities obtained from experiment to experiment (Tables 1 and 2). The purified enzyme (DEAE-cellulose eluate) following freezing in a dry ice - 2-methoxyethanol bath, can be stored indefinitely at -20°C with little loss in activity. However, 12 h after thawing, activity was completely lost at 4°C.

Table 1

Purification procedure for rhodanese from sulfur-grown _T. ferrooxidans_

Fraction	Volume (ml)	NaSCN formed* (μmoles)	Protein (mg/ml)	Total NaSCN formed (μmoles)	Specific activity (μmoles/mg)	Purity (fold)	Recovery (%)
Crude extract	27	0.500	19.0	135.0	0.263	1.00	100
105,000 x g Supernate	27	0.372	12.5	100.0	0.298	1.13	74.4
Supernate, pH 5.0 and 2% streptomycin sulfate	52	0.158	6.3	82.2	0.251	0.95	60.9
0-25% $(NH_4)_2SO_4$ supernate	52	0.150	4.1	78.0	0.366	1.39	57.8
25-90% $(NH_4)_2SO_4$ precipitate	31	0.196	3.0	60.8	0.653	2.48	45.0
DEAE -cellulose eluate	31	0.08	0.08	24.8	10.00	38.02	18.4

*NaSCN formed (micromoles) per minute per 0.1 ml of enzyme fraction. Enzyme activity was determined for 1 min as described in Materials and Methods. The amounts of protein used were: crude extract, 1.90 mg; 105,000 x g supernatant, 1.25 mg; pH 5.0 and streptomycin supernatant, 0.63 mg; 0.25% $(NH_4)_2SO_4$ supernatant, 0.41 mg; 25-90% $(NH_4)_2SO_4$ precipitate, 0.30 mg; DEAE-cellulose eluate 8 μg.

Table 2

Requirements and specificity for rhodanese enzyme from sulfur grown _T. ferrooxidans_

Deletions	Additions	Micromoles NaSCN formed	Micromoles NaSCN per minute (mg/protein)
---	---	1.06	0.236
NaCN	---	0.08	0.018
$S_2O_3^{2-}$	---	0.11	0.024
Enzyme	---	0.07	0.016
Enzyme	Boiled enzyme	0.38	0.084
$S_2O_3^{2-}$	50 μmoles cysteine	0.12	0.027
$S_2O_3^{2-}$	0.1% Mercaptoethanol	0.14	0.031
$S_2O_3^{2-}$	50 μmoles GSH	0.11	0.024

Note: Enzyme activity was determined using a 10-min assay as described in Materials and Methods. Additions and deletions are as indicated. Protein (0.45 mg) of the 90% ammonium sulfate fraction was used.

The enzyme reaction was linear with respect to time up to 10 min, and the reaction was linear with respect to protein concentration up to 16 μg of protein.

Requirements and Specificity of the Reaction

Cyanide, thiosulfate, and enzyme are required for the catalytic formation of thiocyanate (Table 2). Neither cysteine, mercaptoethanol nor reduced glutathione could replace thiosulfate in the reaction. In these and subsequent assays, results represent the average of at least two separate experiments, and reproducibility was demonstrated with different batches of enzyme.

Effect of pH on Enzyme Activity

The activity of rhodanese plotted as a function of pH has a broad pH optimum ranging from pH 7.5-9.0.

Effect of Temperature

Room temperature (25°C) was optimum for the enzyme assay. Above 25°C, enzyme activity rapidly decreased with temperature. However, in the crude 90% ammonium sulfate fraction, boiling for 5 min did not result in complete loss of activity (Table 2), due possibly to some protection afforded by extraneous protein.

Effect of Substrate Concentration

The apparent K_m for thiosulfate and cyanide as determined by the method of Lineweaver and Burk (14) was 5.8×10^{-4}M and 1.1×10^{-2}M, respectively.

Effect of Inhibitors on Enzyme Activity

Table 3 shows the effects of inhibitors on rhodanese. The thiolalkylating reagents iodoacetamide and N-ethylmaleimide inhibited the enzyme by about 85 and 20%, respectively. The chelating agent EDTA and o-phenanthroline had no effect nor did azide, arsenate, arsenite, fluoride, or p-hydroxymercuribenzoate. Sulfite, one of the end products of the reaction, inhibited the enzyme by about 40%. Except for mercury which caused 30% inhibition, metal ions were not tested because they caused the nonenzymatic formation of thiocyanate (15).

Table 3

Effect of various inhibitors on rhodanese activity*

Addition	Concentration (M)	Percentage of control
None	---	100
p-Hydroxymercuribonzoate	10^{-3}	98.9
EDTA	10^{-2}	101.1
Arsenate	10^{-2}	98.9
Arsenite	10^{-2}	101.1
o-Phenanthroline	10^{-3}	100
NaF	10^{-2}	97.7
$HgCl_2$	10^{-3}	71.3
GSH	10^{-2}	95.4
N-ethylmaleimide	10^{-3}	79.3
Na_2SO_3	10^{-3}	63.2
Iodoacetamide	10^{-3}	16.1
NaN_3	10^{-3}	101.1
Mercaptoethanol	0.1%	98.9

*Enzyme activity was determined after 10 min as described in Materials and Methods with additions as indicated. Protein (0.45 mg) of the 90% ammonium sulfate fraction was used.

Isolation and Properties of the Sulfite Oxidase Enzyme

Materials and Methods

Organism and Growth Conditions

Thiobacillus ferrooxidans strain TM was grown on the 9K salts medium (16) supplemented with 0.8 ml of a 0.5 M solution of $FeSO_4 \cdot 7H_2O$ per liter of medium. Colloidal sulfur (0.5%) was added to the salts solution. The culture was grown in three 10-liter quantities of medium contained in New Brunswick Model F-14 fermentors and the medium was steamed at 110°C for 30 min prior to inoculation. The initial pH of the medium was 3.2. The cells were incubated in the fermentors at 28°C with a stirring rate of 150 rpm and an aeration rate of 750 ml/min. The fermentors were inoculated with 5 ml of a suspension containing previously sulfur-grown cells suspended to a final concentration of 10^9 cells/ml; growth was monitored by following a drop in pH to 1.5-1.8. The 30 liters of medium were run through a water cooled Sharples air driven centrifuge and the cells collected, scraped from the bowl and suspended in distilled water adjusted to pH 3 with H_2SO_4. The cells were washed once with the acid water by centrifugation at 4°C in a Sorvall RC2-B centrifuge.

Preparation of Cell-free Extracts and Enzyme Purification.

All procedures were done at 0-5°C. The washed cells were suspended to 10% (w/v) in 0.1 M Tricine-NaOH buffer (pH 8.0) to a total volume of 60 ml and disrupted in a water cooled, 10 Kcycles/sec Raytheon sonic oscillator operating at maximum output. Three separate 5 min treatments were required to break 90% of the cells. The oscillator bowl was cooled for 2 min between each disruption. Phase contrast microscopy was used to monitor breakage of the cells. Cellular debris was removed by centrifugation at 27,000 x g for 20 min in a Sorvall RC2-B centrifuge equipped with a SS-34 rotor. The supernatant was then centrifuged at 105,000 x g for 1.5 h in an International Preparative Ultracentrifuge Model B-60, with a type A-211 rotor. The 105,000 x g supernatant was collected and labeled crude cell-free extract.

The crude extract was applied to a 2.5 x 40 cm DEAE-cellulose column equilibrated with 0.02M Tricine-NaOH buffer at pH 8.0. The sulfite oxidase enzyme ran with the solvent front (Tricine-NaOH buffer) and was collected in about the same volume (60 ml) as had been applied to the column. The eluate was then concentrated to a 10 ml volume by ultrafiltration through an Amicon Model 52 Ultrafiltration Cell with a Type XM-50 filter. The residue retained by the ultrafilter was mixed with calcium phosphate gel (4.0 mg/mg protein) and stirred for 30 min. The gel was collected by centrifugation and the pellet eluted with 0.15 M Tricine-NaOH buffer (pH 8.0). The purified enzyme was stored at -20°C. Protein was estimated according to the Lowry procedure (17) with crystalline bovine albumin as the standard.

Polyacrylamide Disc Gel Electrophoresis - The standard disc gel electrophoresis procedure of Davis (18) was used to check the purity of the enzyme. Protein from each purification step was electrophoresed in triplicate in 6.0% polyacrylamide gel. The protein preparations were layered on the lower gel in 5% sucrose and electrophoresed in a Poly-Analyt disc electrophoresis apparatus (Buchler Instruments, Inc.,Fort Lee, N. J.). The upper buffer was 0.042 M Tris-Glycine (pH 8.9 at 25°C) and the lower buffer was 0.12 M Tris-HCl (pH 8.1 at 25°C). The current was maintained at 2.5 mamp/gel until the bromophenol blue tracking dye reached about 1-2 mm from the bottom of the gel. The gels were removed, fixed in 20% trichloroacetic acid (TCA) for 20 min, stained for 45 min with Coomassie Blue (1% aqueous solution diluted 1:20 with 20% TCA) and then destained with 10% TCA.

The stained gels were scanned at 500 nm in a Linear Gel Scanner (Model 2410, Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Enzyme Assay - The sulfite oxidase was assayed using a modification of the procedure of Charles and Suzuki (10). The standard reaction mixture contained: 0.1 mmole of Tricine-NaOH buffer at pH 8.0, 1.5 μmole of $K_3Fe(CN)_6$, 35 μmoles of Na_2SO_3 (low in PO_4^{3-}) in 5 mM EDTA, enzyme and double distilled water to 3.0 ml. The enzyme reaction was followed as the decrease in absorbance at 420 nm in a Perkin-Elmer Model 124 dual beam spectrophotometer, using the absorbance scale expander.

Molecular Weight Determinations

The molecular weight of the sulfite oxidase was determined using polyacrylamide gel concentrations of 5.5, 6.0, 6.5 and 7.0% according to the method of Hedrick and Smith (19). The gels were fixed, stained, scanned and the R_m's measured. The R_m values were plotted against gel concentration and the slope of the line was determined using a computer program written by Mr. Amnon Liphshitz (Department of Biology, Syracuse University). The molecular weight was determined from a standard curve of proteins of known molecular weight (Liphshitz, A. and Lebowitz, J.: Biophysical Society Abstracts, p. 70a, 1971).

All procedures described were conducted at least 3 times on 3 separate batches of cells grown on sulfur. The reproducilibity of the procedures was good and the R_m of the purified enzyme on the polyacrylamide gel electrophoresis was the same in each case.

Determination of Labile Iron

Labile ferrous and ferric ion were determined on 1 mg of the partially purified enzyme using the procedure of Suzuki and Silver (20).

Reagents

N-tris(hydroxymethyl)-methyl glycine (Tricine), Horse heart cytochrome c, type III, EDTA (Na_4) were obtained from Sigma Chemical Co.; $K_3Fe(CN)_6$ from J. T. Baker Chemical Co.; Na_2SO_3 (low in phosphate), TCA, 2,2'-dipyridyl and colloidal sulfur from Fisher Scientific Co.; Polyacrylamide gel reagents from Canal Industrial Corp.; $Ca_3(PO_4)_2$ gel from Nutritional Biochemicals Corp.; DEAE cellulose from Schleicher and Schull, Inc. All reagents were prepared in double glass distilled water.

Results

Purification of Sulfite Oxidase

The purification results for sulfite oxidase are given in Table 4. To check for purity, after each step in the purification scheme, polyacrylamide disc gel electrophoresis was done on 160 μg of protein from the extract

Table 4

Purification of sulfite oxidase from *T. ferrooxidans* grown on sulfur

	Total Protein mg	Total Units (µmoles $Fe(CN)_6^{3-}$ reduced/hr)	Units/mg protein	Fold pure
105,000 x g supernate	317	317	1.0	1.0
conc. DEAEC eluate	75	104	1.4	1.4
$Ca_3(PO_4)_2$ eluate	7.2	53	7.3	7.3

for each step. Comparative scans of the Coomassie blue stained gels showed a single major peak from the $Ca_2(PO_4)_3$ eluate extract. This peak was determined, by triangulation, to be 75-85% of the protein present. This major band was cut from unstained gels, eluted in 0.05M Tricine-NaOH buffer and enzyme activity determined; the remaining protein was re-electrophoresed and only one band was noted. An ultraviolet absorption spectrum of the enzyme revealed only one peak at 270 nm indicating that the protein must have been contaminated with some nucleic acid. Attempts at trying to stain the gels for sulfite oxidase activity by the method of Fine and Costello (21) were unsuccessful. This was probably due to the electronegativity of the sulfite which reduced the phenazine methosulfate almost instantaneously thus precipitating the nitroblue tetrazoleum.

Analysis for labile iron associated with the partially purified enzyme using the 2,2'-dipyridyl method (20) revealed no ferrous of ferric ion associated with the enzyme.

Enzyme Stability

The 7.3 fold pure sulfite oxidase was stored routinely at -20°C with no appreciable loss of activity upon thawing. The enzyme lost all activity by boiling for 15 min. The enzyme was stable for 8 h at 3°C. Enzyme activity was linear with protein concentration to about 250 μg.

Molecular Weight Determination

The purified enzyme was electrophoresed in triplicate at four different gel concentrations as described. The molecular weight was determined to be approximately 41,500.

Electron Acceptors

Sulfite oxidase could couple to either $Fe(CN)^{3-}$ or oxidized horse heart cytochrome *c*. The K_m for sulfite using $Fe(CN)_6^{3-}$ as the electron acceptor was 0.538 mM. The K_m for sulfite was cytochrome *c* as electron acceptor was 0.578 mM. The K_m for $Fe(CN)_6^{3-}$ was determined to be 250 μM. The requirements for enzyme activity are seen in Table 5.

Table 5

Requirements for sulfite oxidase from *T. ferrooxidans*

Additions or Deletions	ΔA_{420}/min x 10^3
Complete	19
minus SO_3^{2-}	0
minus enzyme	10
+boiled enzyme (15 min)	10
+TCA precipitated enzyme	10

The assay mixture was as described in Materials and Methods.

The protein concentration was 170 μg.

The Effect of pH and Buffer Concentration

When enzyme activity was determined as a function of pH in 0.1 M Tricine-NaOH buffer, it was found that there was no increase in activity from pH 5.4 to pH 9.0. At pH 10.0 and above, enzyme activity increased and no specific pH optimum could be determined. It was assumed that this phenomenon had no physiological significance, thus all assays were conducted at pH 8.0.

The effect of increasing the Tricine buffer concentration on enzyme activity showed a gradual decrease in activity to 60% at 130 μM. When phosphate buffer was added to the reaction mixture in the place of water, an inhibitory effect was noted which completely inhibited enzyme activity at a level of 40 μM. These data agree with the sulfite oxidase isolated from *T. thioparus* (12) but not with the *T. novellus* enzyme (10).

Effect of 5'-AMP

A major reaction involving sulfite oxidation is dependent upon the condensation of 5'-adenosine monophosphate and sulfite to form adenosine-5'-phosphosulfate (APS). The reaction is catalyzed by APS reductase (22).

The effect of 5'-AMP upon partially purified sulfite oxidase from *T. ferrooxidans* was tested. When 1.5 μmole of 5'-AMP was added to the reaction mixture, no effect on enzyme activity was noted.

Discussion

Explaining the mechanism of sulfur utilization in *T. ferrooxidans* was the objective of the present studies. The presence of the rhodanese enzyme in sulfur grown cells indicates that thiosulfate can be split to form sulfite and a sulfur compound. The presence of the sulfite oxidase indicates that the sulfite can be oxidized to sulfate with the production of ATP energy for cell growth.

T. ferrooxidans is similar to other thiobacilli (5, 6, 7, 8) in possessing a rhodanese with a fairly broad pH optimum (pH 7.5-9.0). The inhibition of rhodanese activity by iodoacetamide and N-ethylmaleimide indicates the involvement of thiol groups at the enzymes active site. The K_m for thiosulfate and cyanide compares favorably to that reported for rhodanese from *Chromatium* (23). However, the unusually high value for cyanide (K_m of 10^{-2}M) indicates that this compound is not the physiological acceptor of the sulfur atom split from thiosulfate. Other experiments with mammalian rhodanese (24) indicate that the S acceptor molecule is dihydrolipoate. Dihydrolipoate was not used in the present study.

The sulfite oxidase from *T. ferrooxidans* grown on colloidal sulfur was partially purified and comprised about 75-85% of the purified protein. The enzyme could couple to both ferricyanide and cytochrome *c* and exhibited the same apparent K_m for sulfite when either electron acceptor was used. The apparent K_m for SO_3^{2-} of .58 mM is 6.5 times higher than the K_m for sulfite of the *Thiobacillus thioparus* enzyme (12) and 17 times higher than the apparent K_m for sulfite of the *Thiobacillus novellus* enzyme (11). This could be due to the fact that *T. novellus* and *T. thioparus* sulfite oxidases were isolated from cells grown on thiosulfate, whereas the *T. ferrooxidans* enzyme came from cells grown on sulfur. The latter organisms grows slowly on the thiosulfate and the enzyme from the organism grown on thiosulfate was not tested.

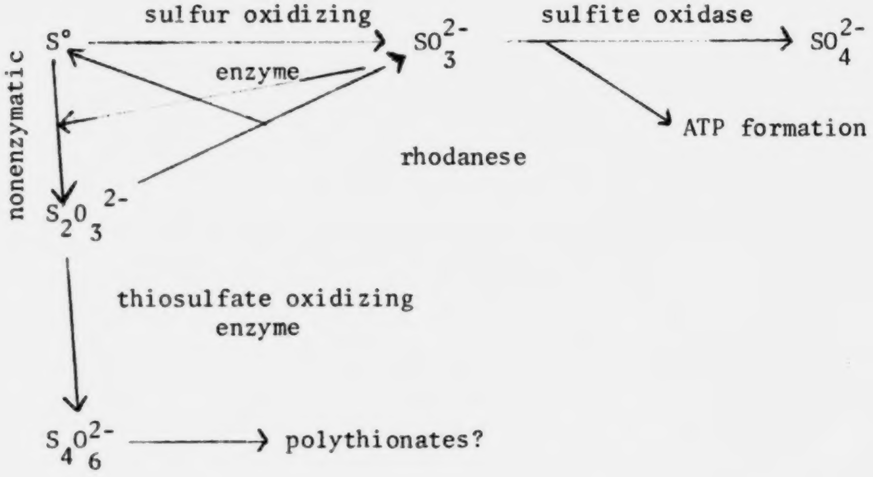
No ferrous or ferric ion was found associated with the partially purified enzyme. The *T. thioparus* (12) enzyme did contain 1 mole of ferrous ion per mole of enzyme and chelating agents inhibited the enzyme activity indicating that the ferrous ion was essential for activity. EDTA and 2,2'-dipyridyl did not inhibit enzyme activity in *T. ferrooxidans*.

The pH optimum of the enzyme was high (pH 10.0) relative to the *T. novellus* (11) and *T. thioparus* enzymes (12), and is probably not the physiological pH optimum. This high pH optimum is interesting since, *T. ferrooxidans* is strongly acidophilic, where the pH of the culture medium prior to harvest was 1.5 to 1.8. It is assumed that this enzyme *in vivo* is not in direct contact with the outside medium. The pH optimum is probably closer to neutrality since Blaylock and Nason (25) have shown, by breaking *T. ferrooxidans* in water, that the intracellular pH is about 4.8-5.0. We can offer no explanation for this pH abnormality for the sulfite oxidase.

The molecular weight of 41,500 was lower than the 54,000 reported for the *T. thioparus* enzyme (12). Whether or not this represents a loss of some enzyme subunit is not known. Tests for purity (gel electrophoresis) did indicate a homogeneous enzyme, but from the ultraviolet absorption spectra (max 270 nm) some nucleic acid contamination is most likely.

Our results indicate that the sulfite oxidase enzyme is the enzyme responsible for energy production in sulfur-grown cells. The enzyme catalyzes the reduction of oxidized cytochrome *c*. It has been shown that the electron transport system in *T. ferrooxidans* most likely involves only cytochrome *c* and cytochrome *a* (25). Thus the electron released from the oxidation of sulfite can pass through this "abbreviated" electron transport chain and ATP could be produced in a manner similar to ATP production in *T. ferrooxidans* when ferrous iron is the energy substrate (25). Reducing power would then be generated by the ATP dependent reduction of pyridine nucleotides (26).

The presence of rhodanese and sulfite oxidase in sulfur-grown *T. ferrooxidans* completes an organized scheme for sulfur metabolism in the cell. The scheme is as follows:


S°
sulfur oxidizing
enzyme
SO_3^{2-}
sulfite oxidase
SO_4^{2-}
ATP formation
rhodanese
nonenzymatic
$S_2O_3^{2-}$
thiosulfate oxidizing
enzyme
$S_4O_6^{2-}$
polythionates?

The sulfur oxidizing enzyme (2) and thiosulfate oxidizing enzyme (3) have been previously identified in sulfur-grown *T. ferrooxidans*. The identification and isolation of rhodanese and sulfite oxidase completes the metabolic cycle which leads to energy formation in sulfur grown *Thiobacillus ferrooxidans*.

SECTION V

STUDIES CONCERNING THE ISOLATION AND IDENTIFICATION OF NEW THIOBACILLUS SPP.

Introduction

Mine drainages which have a pH of 4.5 or above are considered to be alkaline. These drainages, class II, class III, and class IV, can contain appreciable quantities of ferrous iron (0-1000 mg/l) as well as sulfate (500-10,000 mg/l). The interesting question becomes why are these drainages alkaline when they contain ferrous and sulfate? Why have not the iron-oxidizing bacteria developed and oxidized the ferrous to produce ferric precipitates and converted any reduced sulfur compounds to sulfate and acid? These questions prompted the present investigators to look at the microbial content of alkaline mine drainage and to try to find an organism which might be producing some product which keeps the pH from decreasing. From the standpoint of the ecology of alkaline mine drainage, those bacteria present in these drainages might lead to new insights into fundamental situations which exist in a mining environment.

Isolation of an alkaline producing Thiobacillus sp.

Materials and Methods

A sample of alkaline mine drainage (pH 6.8) from an underground mine near Graham, Kentucky, was used to isolate microorganisms by selective culture techniques. The selective medium used was the salts medium of Starkey (27) supplemented with 1% sodium thiosulfate as the energy source. Ten ml of mine water was added to the 90 ml of sterilized thiosulfate medium, which was then incubated on a rotary shaker (150 rpm) for 3-5 days at 28 C. After enrichment, thiosulfate agar plates were streaked with the enriched culture in order to isolate individual colonies of bacteria. In order to select colonies which produce alkaline by-products, phenol red indicator was added to the plates and the presence of alkaline producing colonies was noted as a color change from orange (pH 7.0) to purple (pH 8.0). The alkaline producing organism was subsequently restreaked 4-5 times to determine purity of the organism. This isolate was used for further studies and will be referred to as Thiobacillus strain J since its taxonomic status is currently under study.

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Substrate specificity

Thiobacillus strain J was tested for its ability to grow and produce alkaline by-products. Starkey salts (27) agar plates were prepared with purified, washed agar (Difco) at 2%. Phenol red was added to the liquid agar before solidification. The initial pH's of the plates were adjusted with either H_2SO_4 or NH_4OH. The autotrophic and heterotrophic substrates were added at a 1% concentration. When liquid cultures were used, 50 ml of the salts were contained in 250 ml nephalo flasks with the appropriate energy source added at 1%. All cultures were incubated at 28 C, unless otherwise stated, and liquid cultures were shaken on the rotary shaker at 150 rpm.

Electron microscopy

Metal shadowing of the specimen was done on electron microscope grids. The grids were examined in an RCA 2D electron microscope operating at 50 kv.

Drug sensitivity

Thiosulfate agar plates containing a lawn of organisms were prepared and appropriate drug sensitivity discs (Difco) were aseptically placed on the plates and the plates incubated at 30 C. Zones of inhibition were noted after 3 days incubation.

Mole % G + C

One to two grams (wet wt) of thiosulfate grown *Thiobacillus* strain J was frozen and sent to Dr. Manley Mandel (M.D. Anderson Hospital, University of Texas Medical Center, Houston, Texas) for cesium chloride density gradient centrifugation to determine the mole % G + C of isolated DNA.

Results

Substrate specificity

The alkaline producing isolate, *Thiobacillus* strain J, increased the pH of the medium into the alkaline range after growth on thiosulfate, acetate, citrate, pyruvate, glutamate, yeast extract and nutrient broth (Table 6). A decrease in pH was noted after growth on fructose, lactose, sucrose, glucose and galactose. Formate, thiourea, thiocyanate, dithionite, sodium sulfide, and ferrous sulfate would not support growth of strain J. Growth was not inhibited by 5% NaCl and 0.01% phenol.

Table 6

pH change after growth of Thiobacillus strain J on selected energy sources

Growth Substrate*	initial pH	final pH
$Na_2S_2O_3$	5.9	8.3
Sodium citrate	6.4	9.1
Sodium succinate	6.3	8.7
Sodium pyruvate	6.1	7.8
Sodium glutamate	6.2	8.7
Fructose	6.2	4.7
Lactose	6.1	5.1
Sucrose	6.1	4.6
Glucose	6.1	4.1
Galactose	6.1	3.9
Yeast Extract**	6.1	8.0
Nutrient Broth**	6.1	8.3

*Substrates added to a concentration of 0.5 M.

**Substrates added to 0.5% (w/v).

It was found that strain J would grow on 1% thiosulfate agar plates in a temperature range from 2 C to 41 C. If the pH of the thiosulfate medium were made acidic or basic with H_2SO_4 or NH_4OH, growth occurred at a pH range from 3.3 to 10.0. No visible growth occurred above or below the pH's indicated.

Generation times were determined for strain J on selected autotrophic and heterotrophic substances and are shown in Table 7.

Electron microscopy

Thiobacillus strain J is a gram negative, short rod (2 μm long by 0.5 μm wide) with one polar flagellum as seen by shadow preparation in Figure 1. The surface topology of this organism is similar to typical gram negative bacteria.

Drug sensitivity

When Thiobacillus strain J was tested for drug sensitivity, it was found to be sensitive to Chloromycetin, Kanamycin, Neomycin, Novobiocin, Streptomycin, and Tetracycline (Table 8).

Mole % G + C

The mole % G + C of the DNA isolated from Thiobacillus strain J was determined to be 64.3 with a cesium chloride density of 1.723.

Isolation of an acid producing Thiobacillus sp.

Materials and Methods

Isolation of a new Thiobacillus sp.

An alkaline mine water sample was obtained from the Department of Mines and Mineral Industries of the State of Pennsylvania. The outpourings were from a bituminous coal mine in the Ohio River Valley and had a recorder pH of 7.0.

The water sample was assayed for the presence of iron oxidizing, sulfur oxidizing organisms and heterotrophic bacteria. The former were detected in the standard 9K

Table 7

Generation times of Thiobacillus strain J on various substrates.

Growth Substrate*	Generation Time (h)
pyruvate	0.75
glutamate	1.2
fructose	3.0
glucose	3.7
sucrose	6.5
thiosulfate	7.3

*Substrates added to 1% (w/v) in Starkeys salts medium.

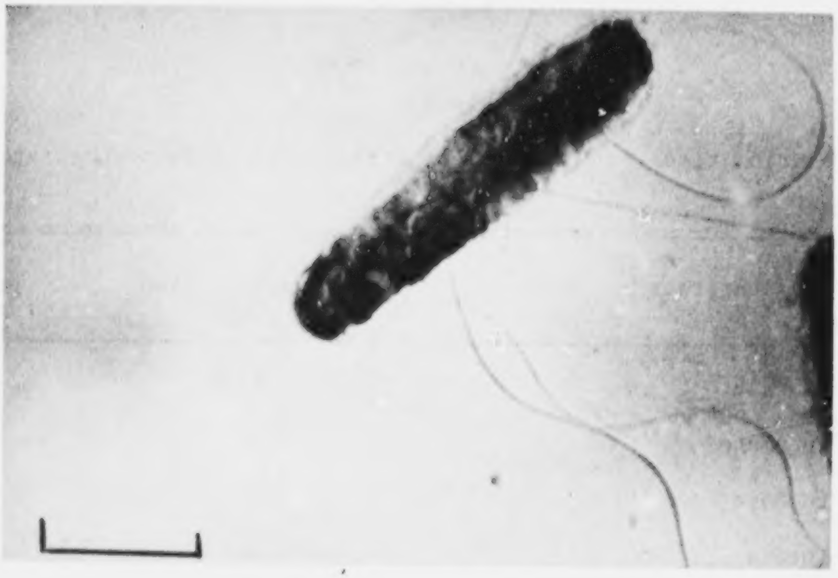

Figure 1. Electron micrograph of a carbon-platinum shadowed cell of Thiobacillus strain J isolated from alkaline mine drainage. Bar marker = 1 μm; 28,000 X.

Table 8

Comparative sensitivity to drugs of _Thiobacillus thioparus_ and _Thiobacillus_ strain J grown on 1% thiosulfate agar plates.

Drug	_Thiobacillus thioparus_	_Thiobacillus_ strain J
Chloromycetin	S*	S
Erythromycin	R	R
Kanamycin	S	S
Neomycin	S	S
Novobiocin	S	S
Penicillin	R	R
Streptomycin	S	S
Tetracycline	S	S

* S = sensitive, R = resistant

medium of Silverman and Lundgren (16) and the heterotrophs on nutrient agar (Difco).

To isolate reduced sulfur oxidizing bacteria from the alkaline mine water, the medium of Starkey was used (27). Thiosulfate was the energy source.

The thiosulfate and phosphates were autoclaved separately and added to the salts aseptically. The final pH of the medium was 7.0. When a solid medium was needed, 1.5% agar was added (Oxoid Agar, Consolidated Laboratories, Inc., Chicago Heights, Illinois). Yeast extract (0.1 and 0.5%) was added to the medium as needed. Also studied was Starkey's medium supplemented with 0.1% glucose and 0.1% sodium glutamate. To detect acid or alkaline production, 0.02% phenol red was added to the medium. All media were autoclaved for 15 min at 121°C and appropriate additives combined as needed.

To obtain an enrichment culture of $Na_2S_2O_3$ oxidizing bacteria, 95 ml of Starkey's medium containing phenol red (pH 7.0) was incubated with 5 ml of mine water and the flask shaken for 7 days on a reciprocal shaker at 28 C. Contents of this flask (5 ml) were transferred to 95 ml of fresh medium contained in 250 ml Erylenmeyer flasks and incubated for an additional 4 days. This flask then served for an inoculum to streak various agar containing plates, where the energy yielding substrates were those described above.

As a result of these studies, two types of thiosulfate oxidizing clones were isolated after 7 days of incubation. These clones were twice re-transferred on different media and their purity confirmed. One organism was an acid producer and the second formed base. The acid producer was readily cultivated on thiosulfate agar and the organism was propagated in liquid medium on thiosulfate to obtain mass quantities of the organism for further studies. This organism appeared to be more predominant in this mine water sample and it was selected for further study.

Growth parameters were examined with the organism grown on thiosulfate; thiosulfate utilization, medium pH changes and cell dry wt were assayed using conventional procedures. Thiosulfate was determined by the colorimetric method of Sorbo (15), pH was measured with a pH meter and cell dry wt was determined by weighing a ml of cell suspension after drying overnight and subtracting the weight of an uninoculated medium control sample treated in an identical manner.

The capacity of thiosulfate-grown cells to oxidize different substrates was assessed manometrically using intact cells. For thiosulfate oxidation the reaction vessel contained 60 μmoles of Tris-HCl buffer (pH, 8.0), 20 μmoles of potassium phosphate, 5 μmoles of $Na_2S_2O_3$ and 4 mg (dry wt) of whole cells. Two tenths of a ml of 20% NaOH was used in the center well and water was added to the reaction vessel to make a final volume of 2.2 ml. Sulfite, tetrathionate and elemental sulfur were also examined in the Warburg flasks. These additions were sodium sulfite, in 5 mM EDTA (5 μmoles), sodium tetrathionate (5 μmoles) and elemental flowers of sulfur (48 mg).

The effects of various organic substrates on sulfite oxidation were also examined; 0.5% of yeast extract, 0.5% of glucose and 0.5% of sodium glutamate were the compounds tested. They were added directly to the Warburg flasks. Oxygen uptake by cells in the presence of yeast extract alone was also tested.

The major cell types isolated were examined under the electron microscope with negative stains (1% ammonium molybdate (pH 5.2)) and shadowed preparations (carbon-platinum).

Results

The acid producing Thiobacillus species was a small gram-negative rod which grew either autotrophically or heterotrophically. Autotrophic growth gave clones which were ropy and yellow-orange in appearance. On an organic medium, they appeared white and mucoid with a raised center. Heterotrophically grown cells were often found in pairs and grew to a comparatively large size. Cells appeared to be motile but flagella were not readily demonstrated (Figure 2, 3). Autotrophically grown cells also appeared to be motile and somewhat smaller than heterotrophically grown cells (Figure 4).

Figure 5 shows the growth parameters of the acid producing isolate propagated on thiosulfate as the energy supply.

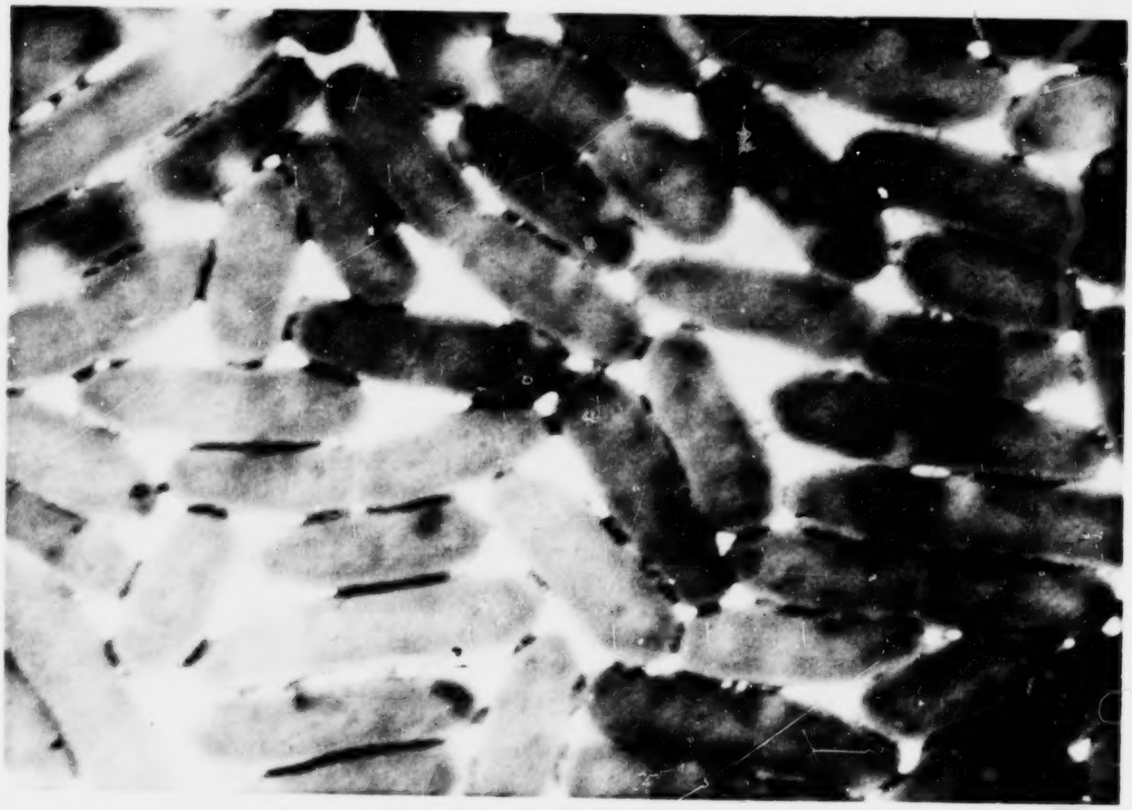

Figure 2. Electron micrograph of carbon-platinum shadowed cells of an acid producing Thiobacillus grown heterotrophically, which was isolated from alkaline mine drainage. 32,900 X.

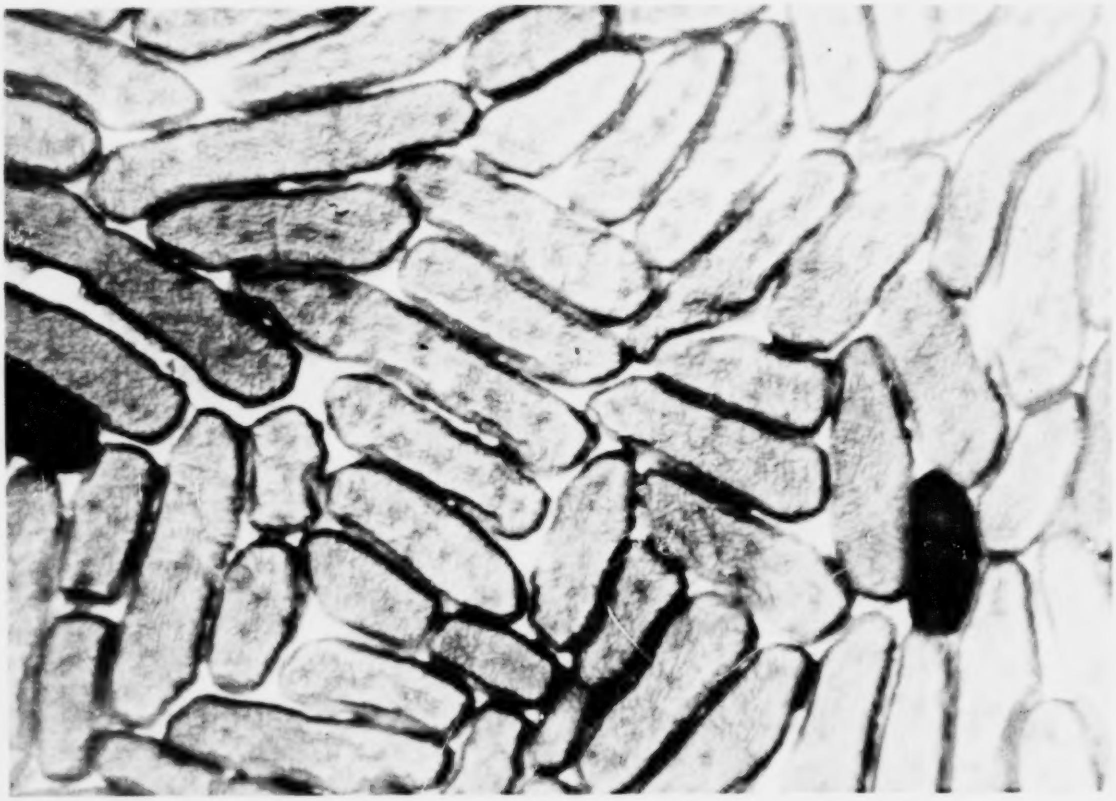

Figure 3. Electron micrograph of negatively stained cells of an acid producing Thiobacillus grown heterotrophically, which was isolated from alkaline mine drainage. 28,200 X.

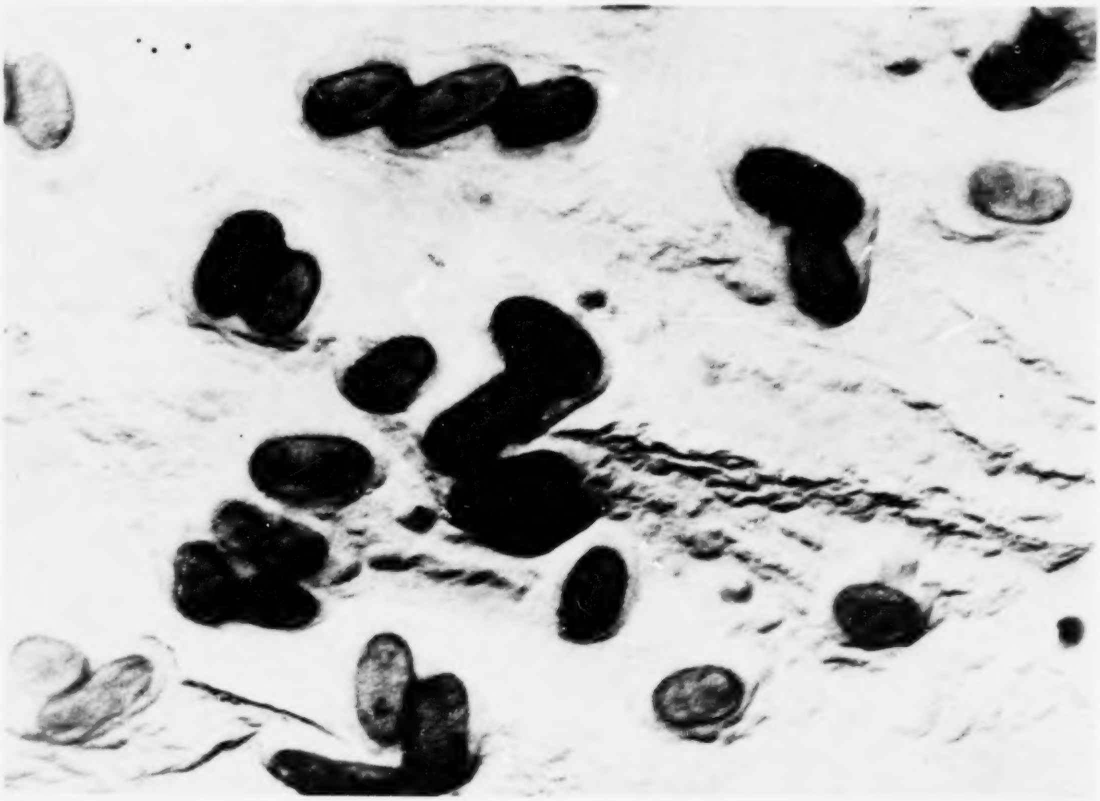

Figure 4. Electron micrograph of carbon-platinum shadowed cells of an acid producing Thiobacillus grown autotrophically on thiosulfate, which was isolated from alkaline mine drainage. 32,900 X.

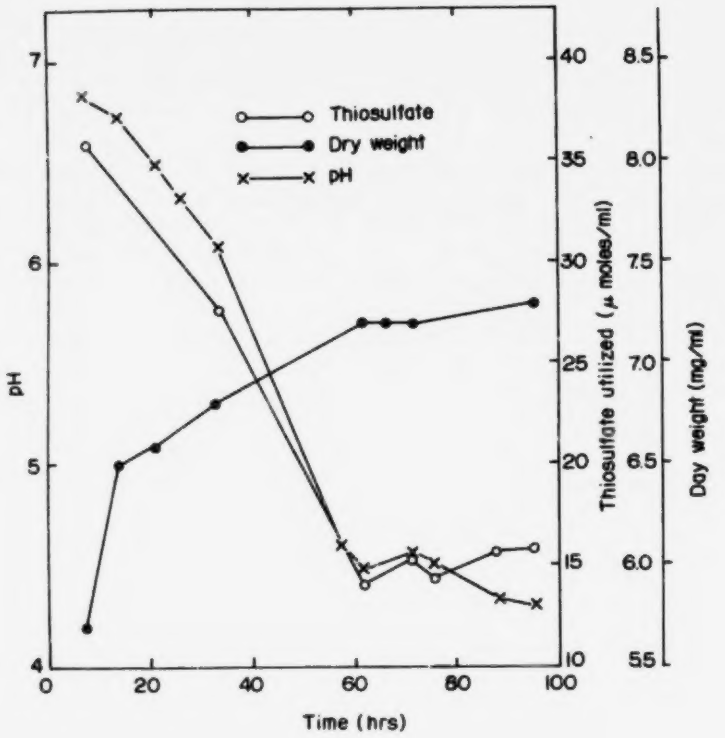

Figure 5. Growth parameters of an acid producing Thiobacillus grown on thiosulfate.

The cell dry wt increased with time and growth leveled off as the substrate was diminished. Thiosulfate was oxidized up to 60 h with time with the concomitant accumulation of acid. The mechanism of oxidation agrees with that suggested for some of the thiobacillus organisms:

$$S_2O_3^{=} + 4O + H_2O \longrightarrow 2SO_4^{=} + 2H^{+}$$

Acid accumulated until a pH of about 4.0; a lower pH appears to be detrimental to the organism. Intact cells of the acid producing isolate also oxidized other reduced sulfur containing compounds (Figure 6). Thiosulfate under these conditions was the preferred substrate with tetrathionate, elemental sulfur and sulfite oxidized in that order. Organic substrates supplemented the oxidation of the reduced sulfur compounds (Figure 7). Yeast extract was by far the better source of nutrient. This result was supported by growth studies in liquid and solid media as well as in manometric studies (Figure 8). Yeast extract, in the absence of any other substrate, was oxidized readily by this organism. Endogenous oxidation represents O_2 uptake by the organism in the absence of any exogenous substrate.

Although alkaline mine water does contain organisms that can oxidize ferrous iron, the aforementioned thiosulfate oxidizing isolate did not oxidize iron during the 7 day incubation period tested using the standard 9K medium.

Discussion

A new *Thiobacillus* species has been isolated and characterized from alkaline mine drainage. The new species, designated at present as strain J, was studied taxonomically to try to relate it to known *Thiobacilli*. From its growth characteristics and substrate specificity strain J resembles *Thiobacillus novellus* and *Thiobacillus intermedius* (28). The sensitivity to the drugs tested indicates that strain J resembles *T. thioparus*. The moles % G + C of the isolated DNA (64.3 mole %) is extremely close to *T. thioparus* and *T. intermedius* (63.3 and 64.8 mole % respectively, M. Mandel, personal communication) indicating a relationship to these two known species.

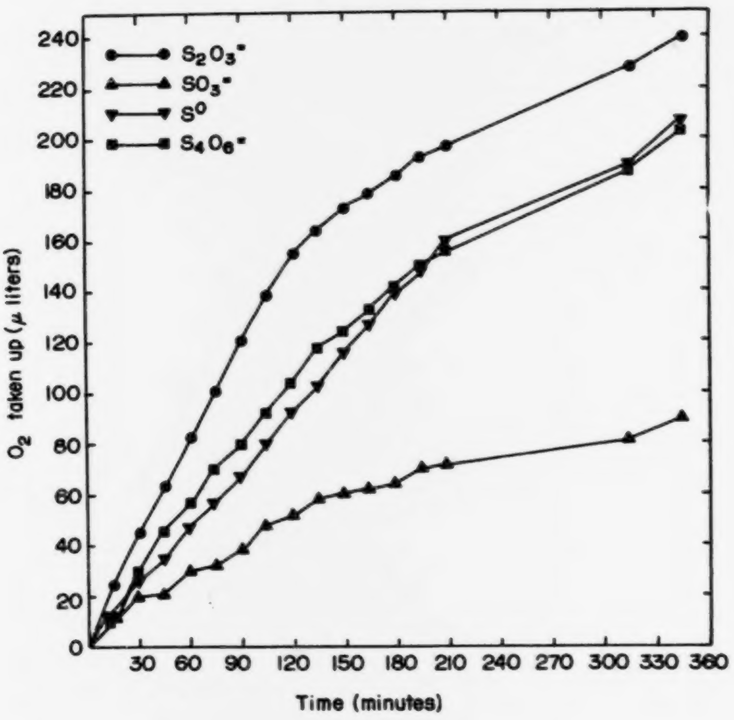

Figure 6. Oxygen uptake, measured manometrically, by the acid producing *Thiobacillus* oxidizing reduced sulfur compounds. Four mg (dry weight) of cells were used in each manometer flask.

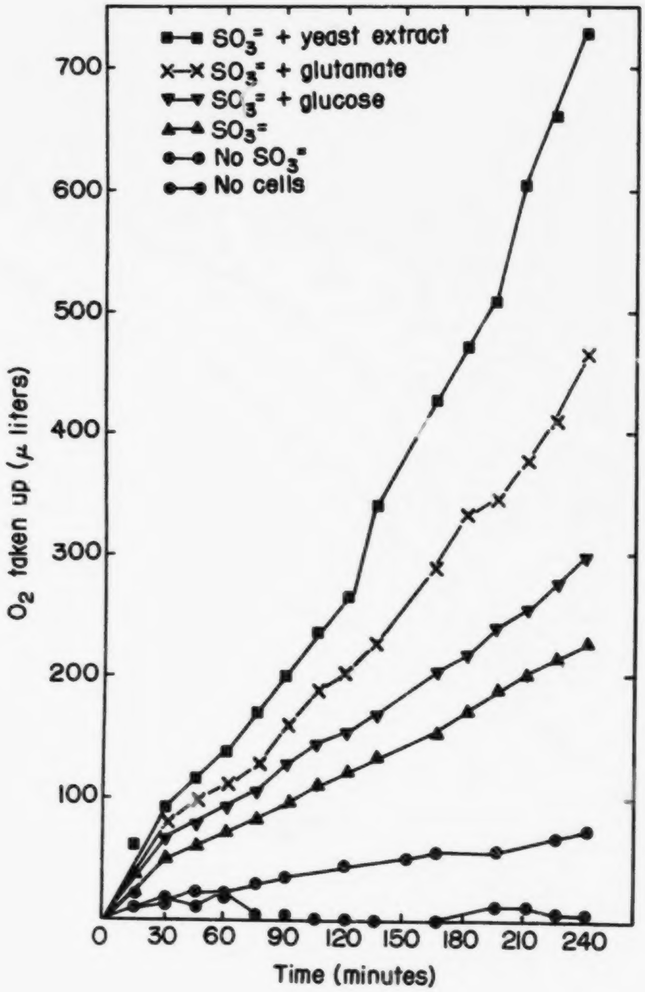

Figure 7. Oxygen uptake, measured manometrically, by the acid producing *Thiobacillus* oxidizing sulfite in the presence and absence of organic compounds. Six mg (dry weight) of cells were used in each manometer flask.

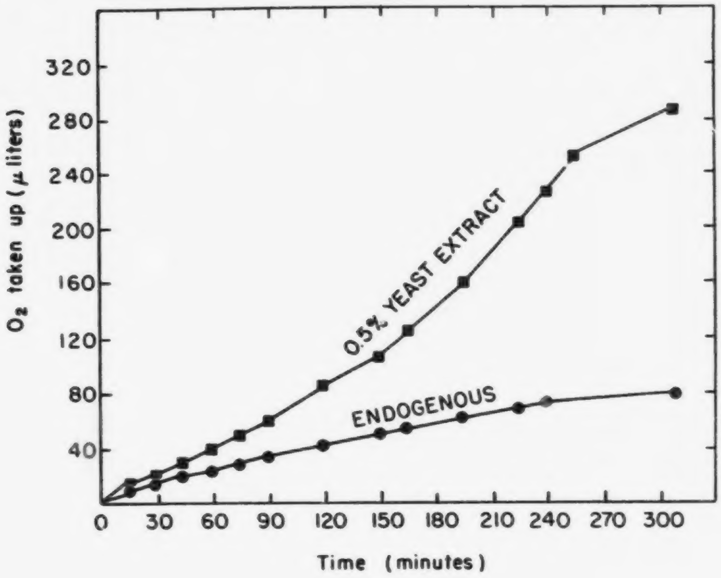

Figure 8. Oxygen uptake, measured manometrically, by the acid producing Thiobacillus oxidizing 0.5% yeast extract. Endogenous (no substrate) is also shown. Two mg (dry weight) of cells were used in each manometer flask.

We feel, however, that there are enough significant differences between strain J and other known *Thiobacilli* to call it a new species. The mole % G + C is significantly different from *T. novellus* (68.4 mole %). Also, *T. novellus* is sensitive to 5% NaCl, 0.01% phenol and will not grow on 0.5% citrate. The mole % G + C of strain J is similar to *T. thioparus*, but *T. thioparus* has not been shown to grow heterotrophically on any of the organic substrate tested here.

The final pH attained after growth on thiosulfate is a rather important and accepted method of differentiating the *Thiobacilli* taxonomically. *T. intermedius* is fairly acidophilic (growth range of pH 2-3) and does not increase the pH of the medium after growth on thiosulfate (28). Also, *T. intermedius* is sensitive to 5% NaCl and will not grow on 0.5% glucose, as strain J will. The final pH attained after growth of *T. novellus* is pH 5.0 (28) which is much lower than that produced from strain J (pH 8.3 on thiosulfate, Table 6). Also, strain J had the ability to grow over a vast range of temperature (2 C to 41 C) and pH (3.3-10.0). Other known *Thiobacilli* are incapable of this. Strain J will not grow anaerobically using nitrate as the final electron acceptor, thus eliminating the possibility that it might be *T. denitrificans*.

The acid producing isolate, studied separately from strain J, was also a gram negative motile, short rod isolated from alkaline mine drainages.

The *Thiobacillus* type isolate forming acid has many features similar to *Thiobacillus intermedius* a facultative autotroph. There were some physiological differences however, notably the failure of the isolate of this study to lower the pH of the medium below 4.0. *T. intermedius* lowers the pH to almost 2 when grown on a thiosulfate medium. Also, *T. intermedius* can oxidize sulfide, a compound which was not tested in the present study. Other than these differences most test parameters employed were similar. *Thiobacillus novellus*, another facultative autotroph, is not motile and is not pH tolerant. It thus seems that the *Thiobacillus* isolated and studied here is either a new species of *Thiobacillus* or a variant of *T. intermedius*.

It is of interest that the natural environment of coal mines established a niche whereby the various thiobacilli can survive and evolve. One would probably expect that since the organisms are closely related and that their separation is by relatively minor physiological differences, the complete spectrum of the organisms composing the genus *Thiobacillus* inhabits mine waters. This naturally remains to be studied further. However, it does seem to be apparent that both types of mine water, acid and alkaline, contain the same thiobacilli but that the predominance of reduced iron and pyrite in acid drainage favors *Thiobacillus ferrooxidans*. The alkaline waters favor the reduced sulfur oxidizers, some of which are not acid tolerant. Whether or not these latter organisms contribute to the alkalinity of the mine waters remains to be demonstrated conclusively in the laboratory.

SECTION VI

STUDIES CONCERNING THE METABOLISM OF *THIOBACILLUS FERROOXIDANS* GROWN ON IRON

Introduction

When considering the growth of *T. ferrooxidans*, either in acid mine drainage or under laboratory conditions, it is of importance to consider the structure and function of the outer layer which surrounds the cell. It is this outer layer which is in contact with the aqueous acidic environment, and it would be reasonable to assume that this layer is important in maintaining a balance between the acidic environment (pH 2.5-3.5) and the less acidic cytoplasm (pH 4.8-5.0 to above (25)). The general anatomy and fine structure of *T. ferrooxidans* has been previously described (29). However, the structure of the cell envelope layer has not been described in detail until now. The first part of this section describes the fine structure and chemical composition of the lipopolysaccharide outer layer of *T. ferrooxidans* grown on iron.

The study of the importance of the outer layer of the cell as it relates to maintaining a pH balance can also be coupled to the study of the site of iron oxidation. In order to determine, at the molecular level, where and how iron oxidation occurs, it would be beneficial to have a rather accurate kinetic assay procedure for determining rates of iron oxidation by whole cells. In the second part of this section, a description of a new and improved method of studying the rates of iron oxidation by whole cells is described.

Another aspect of iron oxidation by *T. ferrooxidans* is the study of energy production. As previously described, ATP, the cells energy molecule, is produced in the cell as the electron released in ferrous oxidation moves through the electron transport system. The ATP molecule can undergo many reactions which are essential to cell growth. When the ATP is broken down to release energy, sometimes the "high energy" molecule pyrophosphate is formed. In order to convert this pyrophosphate molecule to two orthophosphates, which are necessary for ATP synthesis, the inorganic pyrophosphatase enzyme carries out the necessary reaction. This reaction is necessary to maintain the balance between ATP, ADP and AMP in the cell, the so-called "ATP charge" concept. The inorganic pyrophosphatase enzyme was studied in *T. ferrooxidans* grown on ferrous iron. The enzyme had not been previously reported in chemoautotrophs until the present work was done, which is reported in the third part of this section.

The structure and chemical composition of the cell envelope

Materials and Methods

Cell culture.

Thiobacillus ferrooxidans was propagated in 9K medium in 5-gal carboys under forced aeration at 28 C for 54 hr and harvested as described by Silverman and Lundgren (16). Cells at this time are in the late log phase of growth; approximately 6 g (wet weight) of cells are obtained from 96 liters of medium. Cell numbers are about 2×10^8 cells/ml and have a generation time of about 8 h.

Electron microscopy

Cells (as suspensions), peptidoglycan (PG) and lipopolysaccharide (LPS) to be examined as thin sections were fixed with 1.5% glutaraldehyde in S-collidine-hydrochloride buffer (0.05 M, pH 7.6) for 10 min at room temperature, washed once (for 10 min) with the same buffer, and then fixed overnight at room temperature in osmium tetroxide (1.0%) in distilled water (pH 6.2). The aforementioned treatments were selected based upon results of different trials. These appeared to give the best results. The osmolality of the fixative was 180 milliosmal, as calculated by using the method of Maser, et al (30). The fixed cells were embedded in 2.0% agar prepared with distilled water, and the agar was cut into 1-mm cubes. The embedded cells were dehydrated with ethyl alcohol (5 to 100%) and embedded in Epon 812 by the method of Luft (31). Sections were cut on an ultramicrotome with glass knives and then stained with 1.0% uranyl acetate (pH 4.5) for 30 to 60 min at 60 C, followed by lead citrate (32) for 5 min at room temperature. Purified LPS suspended in distilled water was also examined by negative staining by using methods previously described (33). Thin sections and negative stain preparations were examined in either an RCA EMU-2D or in a JEM-7 electron microscope.

Extraction and purification of lipopolysaccharide.

LPS was extracted and purified by the classical method of Westphal (34).

After centrifugation for 30 min at 3,000 x g, the aqueous upper phase was withdrawn, an equal volume of distilled water (preheated to 75 C) was added to the remaining phases, and the extraction was repeated. The aqueous phase was again withdrawn, pooled with the initially extracted layer and dialyzed against running tap water for 6 h and against three changes of distilled water for 20 h. The dialyzed aqueous phase LPS was then lyophilized and weighed.

Lyophilized LPS (50 mg) was dissolved in 7.5 ml of distilled water containing 0.75 ml of 2% hexadecyltrimethylammonium bromide (Cetavlon, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.); the solution was stirred at room temperature for 15 min before centrifuging at 3,000 x g for 30 min to partially remove ribonucleic acid (RNA; 35).

RNA associated with LPS was estimated to be 3% by assuming that absorbancy at 260 nm was due to nucleic acid and that 50 μg of RNA has an optical density (OD) of 1.0. The supernatant extraction was collected, lyophilized, and dissolved in 3.0 ml of 0.5 M NaCl. The LPS solution was then added to 10 volumes of absolute ethanol and held at 0 C for 1 h until a flocculant precipitate formed. The ethanol mixture was centrifuged, and the pellet was collected and suspended in 4 ml of distilled water. The solution was dialyzed for 30 to 48 h against several changes of distilled water in the cold. The dialyzed preparation was lyophilized and stored in a dessicator until use. Different batches of LPS extracted from *T. ferrooxidans* as described above were pooled and used for the subsequent experiments.

Relative density and sedimentation velocity of LPS.

The relative density of LPS was determined by moving zone centrifugation by using sucrose. Centrifuge tubes containing 50.9 to 68.0% sucrose were prepared in a total volume of 3.6 ml and allowed to equilibrate overnight. LPS (1.0 mg/1.2 ml of water) was placed on top of the gradient and centrifuged at 60,000 x g for 90 min at 20 C. After centrifugation, LPS was visible as a slight band in the gradient.

The sedimentation velocity pattern of LPS was obtained by dissolving 4 mg of LPS in 1.0 ml of 0.066 M phosphate buffer (pH 7.1). The solution was placed in a double sector cell and centrifuged at 20,410 rev/min for 33 min in a Spinco model E analytical ultracentrifuge. Photographs of schlieren optic patterns were taken on metallographic plates (2 x 10 inch plates, high green contrast, Eastman Kodak Co., Rochester, N. Y.) at various time intervals. All photographic measurements were made on a Nikon enlarger (Nippon, Kogaku, K. K., Japan) with a calibrated stage micrometer.

Chemical analyses of LPS.

The phosphorus content of LPS was determined by the method of Chen, et al (36), and Taussky and Shorr (37). Total nitrogen was determined by converting the organic nitrogen in 1 mg of lyophilized LPS to NH_3 by acid digestion and determining NH_3 with Nessler's reagent (38). Iron was determined by the method as described by Suzuki and Silver (20). Magnesium and calcium were estimated by atomic absorption spectrophotometry (Analytical Methods for Absorption Spectrophotometry, Perkin-Elmer, Norwalk, Conn.).

Heptose was determined by the procedure of Dische (39) as modified by Osborn (40). Hexose was determined by a modified anthrone method by using a 2% solution of anthrone in ethyl acetate (41). The method of Aminoff, et al (42) was used to determine 2-keto-3-deoxyoctulosonic acid. Hexosamine was determined by the method of Rondle and Morgan (43). Glucose and galactose were determined by using glucostate kits, respectively (Worthington Biochemical Corp., Freehold, N. J.).

Gas-liquid chromatography. An F & M model 500 gas chromatograph was used equipped with a hydrogen flame ionization detector and 6-ft, U-shaped, 3% SE-30 column (60 to 90 mesh). The separation was done at 180 C. Sugars were first converted to methyl esters without directly determining yield; 2 mg of LPS was suspended in 20 ml of dry methanolic 0.5 N HCl and methanolysis was done at 80 C for 48 h in a screw-cap test tube provided with a Teflon liner. Lipid released during methanolysis was removed by extraction with an equal volume of hexane. The extraction was repeated three times. HCl was removed by repeated evaporation of the sample to dryness under reduced pressure. The dried sample was taken up in 2 ml of methanol and passed over a column of Amberlite IR-120 resin in the H form which was prewashed with methanol. Pooled washings were evaporated to dryness under reduced pressure and redissolved in 1 ml of methanol. The final solution was dried under a gentle stream of air. Sugar-O-trimethylsilyl (TMS) derivatives were prepared by the addition of 1 ml of dry pyridine, 0.1 ml of trimethyl chlorosilane, and 0.1 ml of hexamethyldisilazane. The mixture was shaken vigorously for 5 min; 1 to 3 μliters was used for gas chromatographic analysis (44).

Results

Electron microscopy of the cell envelope.

Figure 9 shows a thin-section profile of normal iron-grown cells possessing a multilayered cell envelope typical of gram-negative bacteria and in particular, similar to the thiobacilli (45, 46). A detailed description of the organization of the layers in the envelope of the iron-oxidizing chemoautotroph has been given (29).

Chloroform-methanol extracted cells show an almost complete removal of the outer layers of the envelope (Fig. 10) except where two cells are firmly attached and apparently protected from the solvent (Fig. 11). The chloroform-methanol extraction removes LPS as determined by chemical analysis. The densely stained PG layer of the cell is still intact after chemical extraction and is identified in these micrographs based upon comparison to normal cells and on previous results of chemical extraction studies (33). Also noted in Fig. 10 and 11 are translucent areas surrounding the polyhedral bodies seen in untreated cells (Fig. 9). The translucency is believed due to materials lost by solvent extraction. The solvent extraction seems to have damaged the cytoplasmic membrane but this is difficult to prove for even untreated cells are difficult to fix properly. Proper fixation of thiobacilli is a general problem which is still not solved (45, 46).

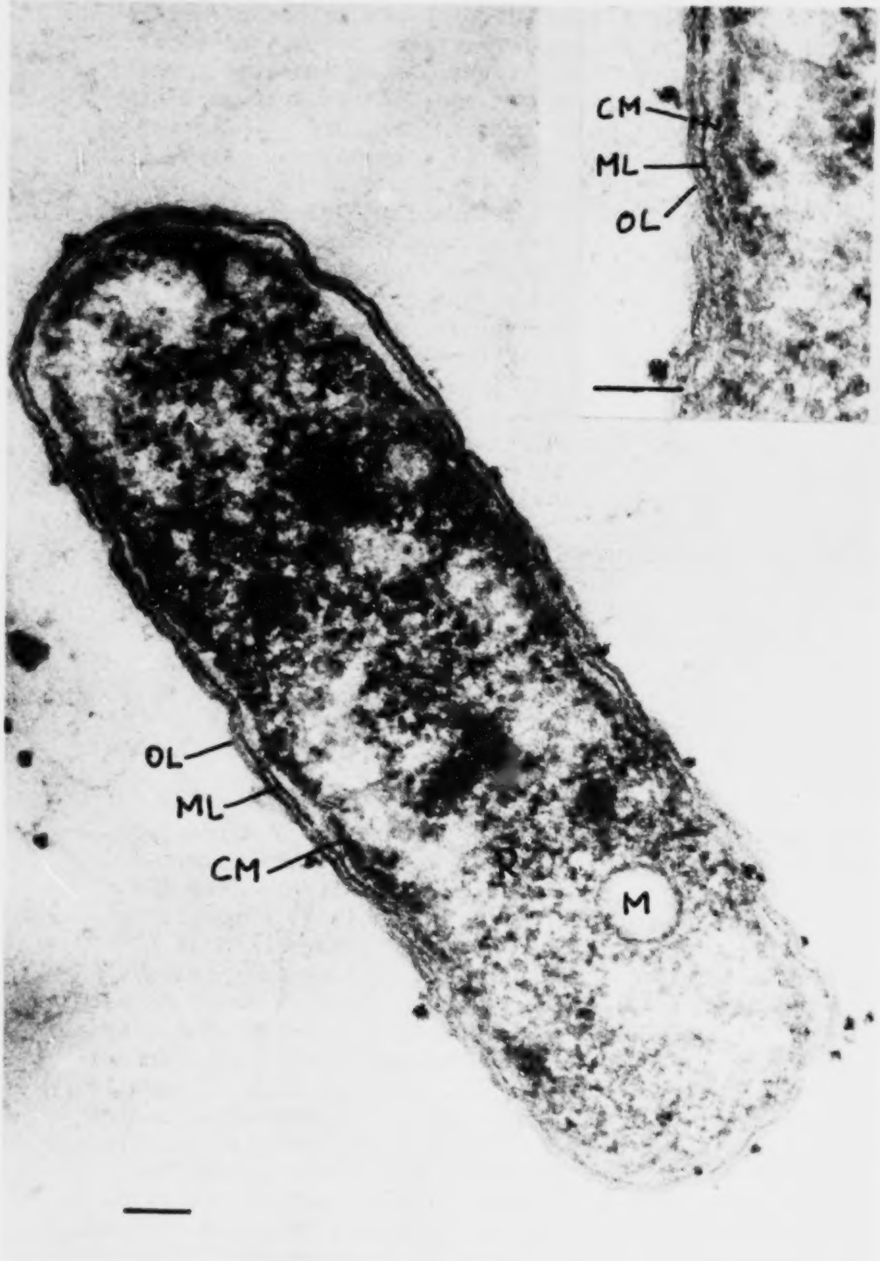

Figure 9. Thin section of a T. ferrooxidans cell grown autotrophically on iron showing a multilayer cell envelope. The labels are outer layer (OL) or lipopolysaccharide (LPS); middle layer (ML) or peptidoglycan (PG); cytoplasmic membrane (CM); ribosomal particles (R); and membrane vesicle (M). Bar marker represents 0.1 μm.

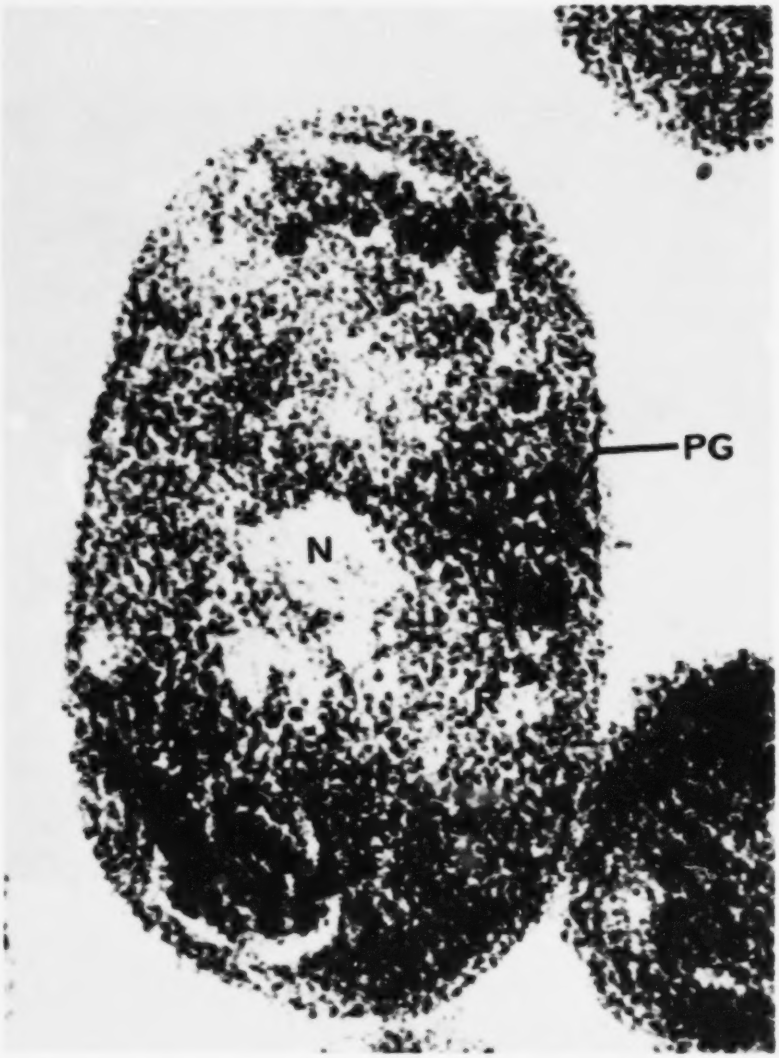

Figure 10. Thin section of T. ferrooxidans after chloroform--methanol extraction. Nucleus (N), ribosomes (R) and peptidoglycan (PG). The bar marker represents 0.2 μm.

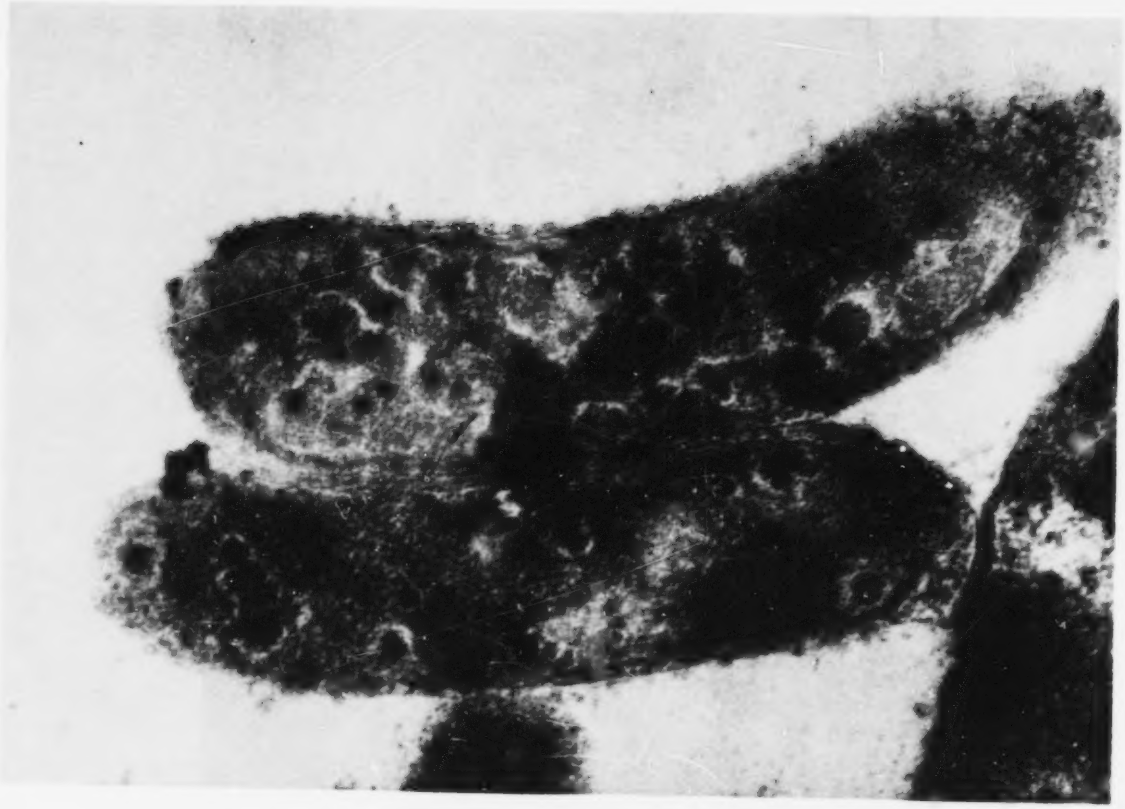

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Figure 11. Thin section of T. ferrooxidans treated as in Fig. 10.

When phenol-extracted LPS was embedded, sectioned, and examined in the electron microscope (Fig. 12), typical unit membrane structures were seen with an approximate width corresponding to that observed in thin sections of intact cells. The triple-layered structures are generally arranged in elongated and coiled formations of various sizes. The clearly resolved areas of the section of LPS have a measured thickness of about 8 nm. A negatively stained preparation of purified LPS is shown in Fig. 13. The large perforated sheetlike structures and ribbon networks are typical of phenol-extracted LPS from *Salmonella typhimurium* (47). No explanation is available for the holes in the sheets but this appearance was consistent for many preparations.

Physical and chemical properties of LPS.

LPS extracted by phenol-water accounted for approximately 4 to 6% of the dry weight of the cells. The amount of contaminating nucleic acid initially present was estimated to be 3%; this was reduced to 1.6% after Cetavlon precipitation. This level of contaminating RNA agrees with the level of nucleic acid in purified LPS described by Burton and Carter (48).

The LPS has a relative density of 1.28 based on results of sucrose gradient centrifugation and was visible as a single band in the 59.4% sucrose fraction. The LPS had a sedimentation coefficient of 99.9S (uncorrected) and an $S_{w,20}$ of 105 when corrections were made for the viscosity and density of the buffer (Fig. 14). The latter value was based on a calculated partial specific volume (V) of 0.79 cm^3/g for the LPS (derived from its relative density of 1.28). The concentration dependence of the sedimentation coefficient was not checked.

Results of chemical analyses of LPS for sugars are shown in Table 9. Fig. 15 shows a profile of a gas-liquid chromatogram of the sugar components. The assignment of each sugar to a particular peak is based on the retention time of an α-methyl-D-glucoside standard (Pfanstiehl Chemical Co., Waukegan, Ill.) given an arbitrary value of 1.0. The brackets depict the α and β isomers of the sugars. The sugar components identified in the LPS are similar to those found in the LPS isolated from gram-negative heterotrophs (49). Nitrogen and phosphorus were present in LPS as was iron which was mostly

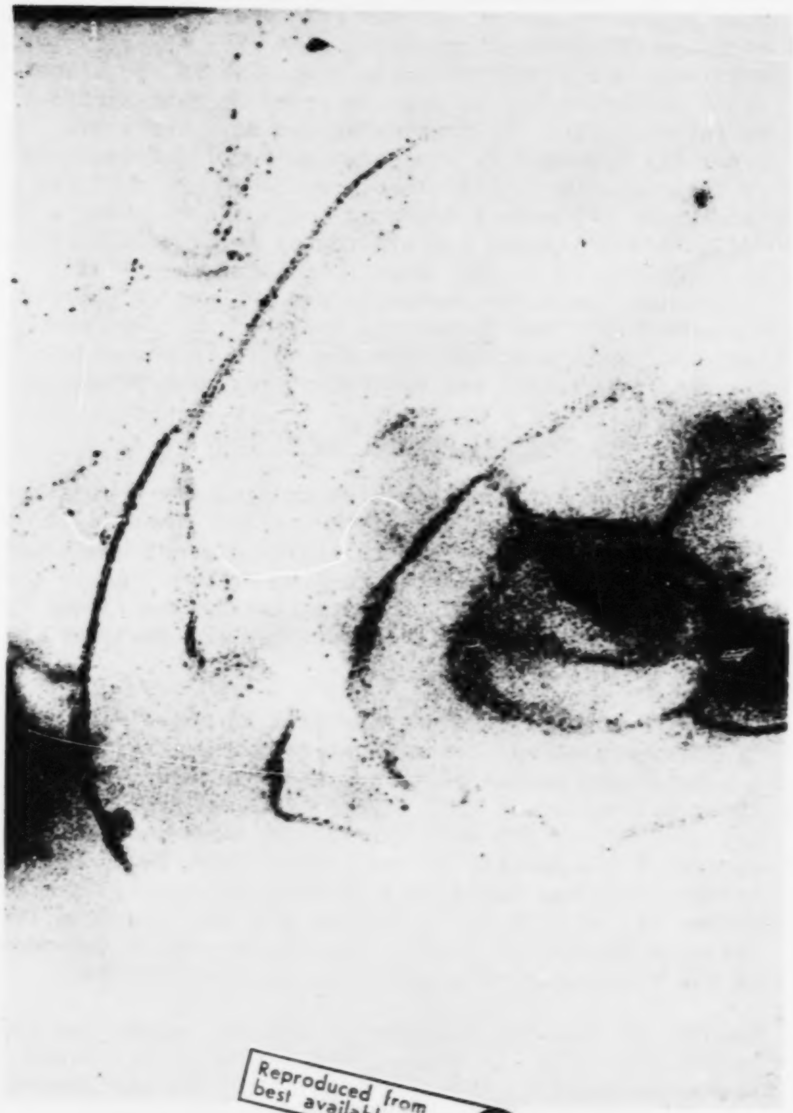

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Figure 12. Thin section of purified LPS from T. ferrooxidans showing the membrane-like tripartite structure (12 nm).

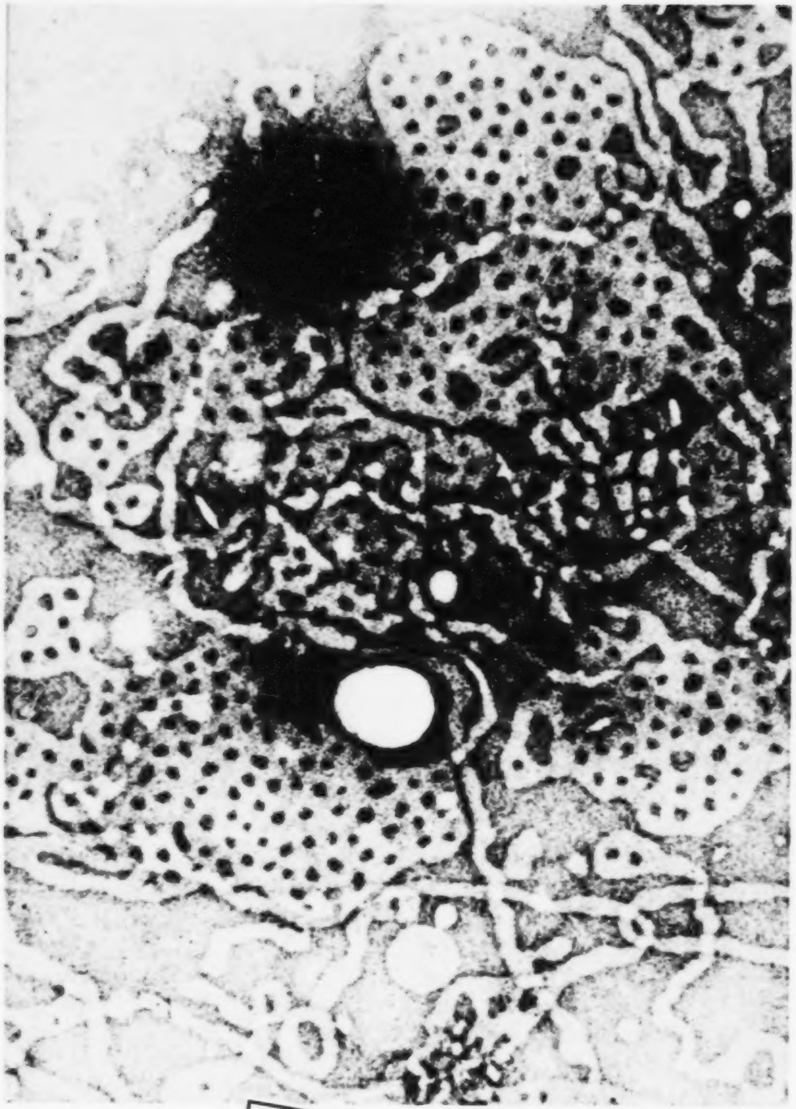

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Figure 13. Negatively stained purified LPS from T. ferrooxidans showing the ribbons and sheets of LPS.

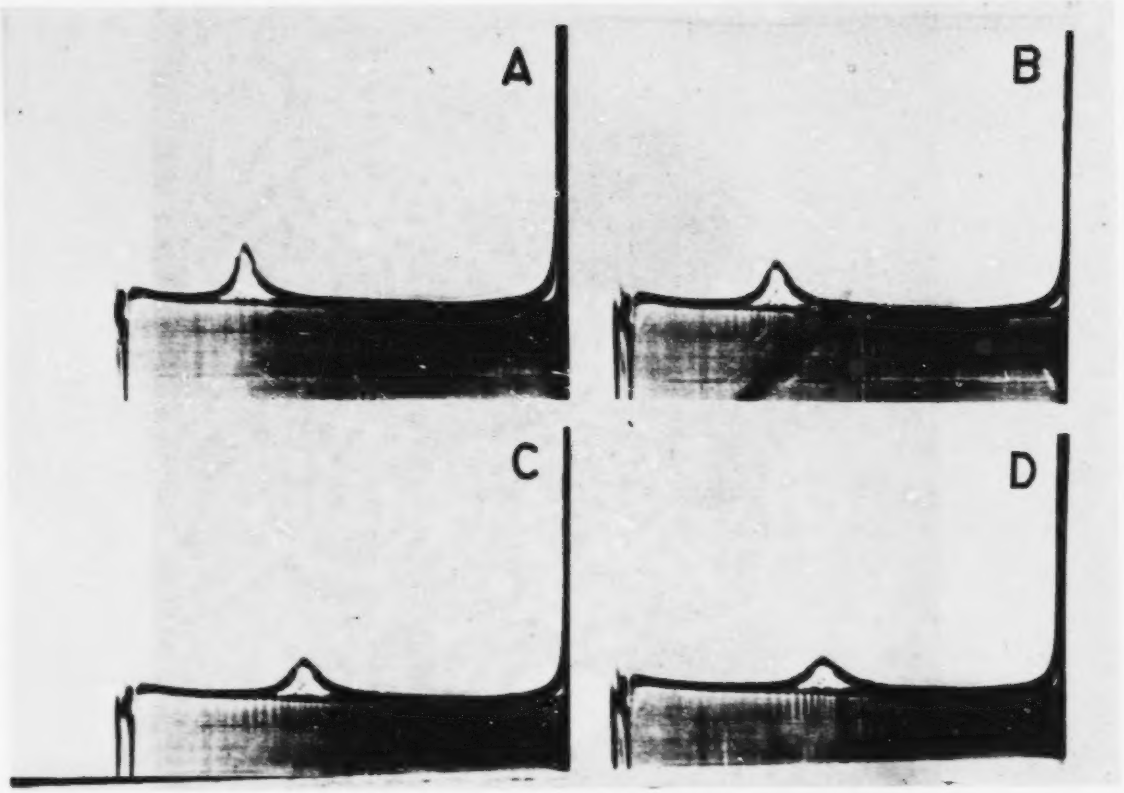


Figure 14. Sedimentation velocity pattern of phenol extracted LPS from T. *ferrooxidans*. The sample was centrifuged at 20,410 rev/min at 20 C. Approximately 4 mg of LPS was dissolved in 0.066 M phosphate buffer (pH 7.1). Time (min) after attaining speed: (A) 16, (B) 20, (C) 24, (D) 28. The bar angle was 65°. The sedimentation coefficient of the LPS was corrected for the viscosity and density of the buffer. The partial specific volume ($\overline{V}$) of the LPS was calculated from the relative density of the macromolecule (1.28), where $1/1.28 = \overline{V}$.

Table 9

Sugar composition of lipopolysaccharide (LPS)
from *T. ferrooxidans*.

Carbohydrate	LPS[a] %
Hexose (total)	39.2
Heptose[b]	12.7
Hexosamine	4.5
2-Keto-3-deoxyoctulosonate (KDO)[c]	8.7
Glucose	3.0
Galactose	6.5

[a] Values of analyses here and in Table 10 represent averages determined from at least three separate determinations which were done on samples of LPS extracted from different batches of cells.

[b] Assumed that when OD_{505} minus OD_{545} = 1.07, then L-glycero-D-mannoheptose = 1.0 mole.

[c] Assumed KDO had a molar extinction coefficient of 72×10^{-3} and a molecular weight of 236.

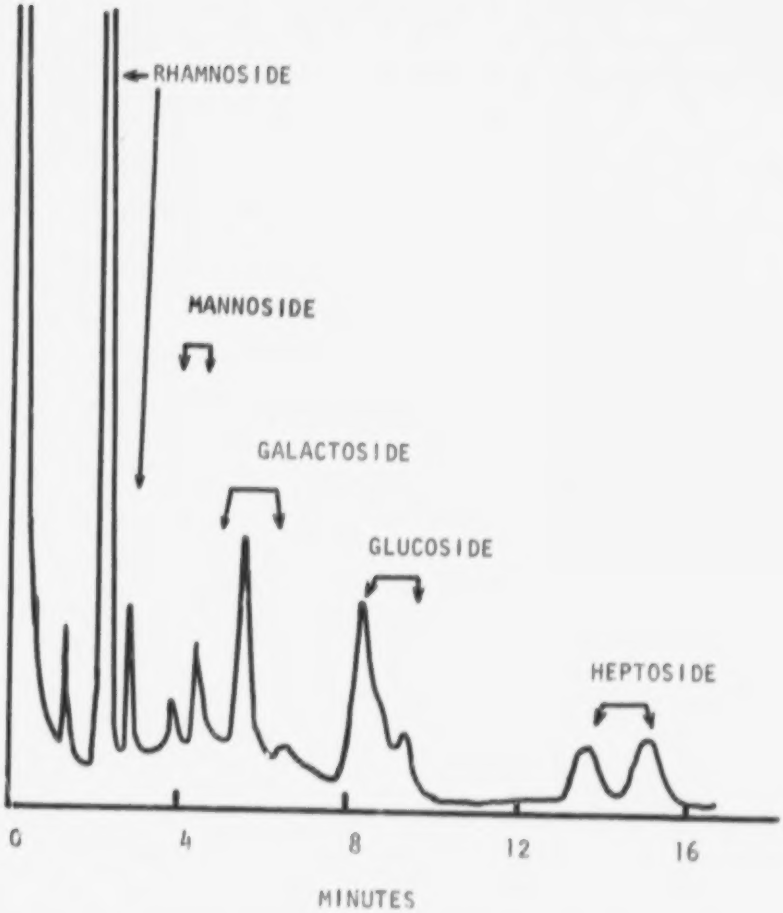


Figure 15. Sugar components of LPS from T. *ferrooxidans*. Sugars were determined by gas chromatography as described in Materials and Methods. One µl of sample was injected, the sensitivity range was 10, and attenuation at 32. No KDO was detected by this method since it is destroyed during methanolysis.

in the ferric form. Other cations present were calcium and magnesium (Table 10).

A new whole cell, iron oxidation assay

Materials and methods

Organism and culture procedure.

T. ferrooxidans strain TM was grown on the 9K medium and in the manner previously described (16) in 16-liter glass carboys under forced aeration. Cells were harvested in a Sharples centrifuge after 48 h of growth, washined three times with distilled water (pH 3.0), and stored in the cold (6 C) in acidified 9K salts or β-alanine-SO_4^{2-} buffer (pH 3.6, 0.01 M). Stored cells were used within 4 days.

Development of an assay system.

Initially, a direct colorimetric assay was used which measured the appearance of small amounts of Fe^{3+}, in the presence of variable anions, as a red complex formed with sodium thiocyanate (2). The reaction was stopped with 5 ml of 2 N HCl prior to analyzing for Fe^{3+}. The addition of HCl to tubes containing Fe^{3+} gave rise to a yellow color, presumably due to the formation of a ferric chloride complex, and it was possible to estimate the color spectrophotometrically at 410 nm. This Fe^{3+}-chloride complex formation served as a basis for the Fe^{3+} assay used in this report.

The assay procedure measuring the appearance of Fe^{3+} was as follows. Five acid-washed and thoroughly rinsed Bellco colorimeter tubes, each containing 3.8 ml of distilled water adjusted to pH 3.0 with H_2SO_4, were placed in a water bath at 35 C; 0.2 ml of a cell suspension (2 mg, dry weight of cells) was added to each tube, and O_2 was bubbled through the suspension via Pasteur pipettes. To each tube, 1 ml of $FeSO_4 \cdot 7H_2O$ solution containing 2.5 mg of Fe^{2+} was added in rapid succession by use of a repeating syringe. The oxidation was stopped at 0, 1, 3, 6, and 10 min by rapidly adding 5 ml of 2 N HCl. The HCl was added before the addition of Fe^{2+} ion for the zero-time tube. Tubes were removed and analyzed for Fe^{2+} at 410 nm in a Bausch & Lomb

Table 10

Elemental analysis of lipopolysaccharide (LPS)

Element	LPS µg/mg
Nitrogen	9.25
Phosphorus	3.89
Mg^{2+}	0.13
Ca^{2+}	1.31
Fe^{2+} and Fe^{3+}	83.00

Spectronic 20 colorimeter. The intensity of the color was directly related to the amount of Fe^{3+} formed and agreed well with Fe^{3+} determinations by the thiocyanate assay method.

Kinetic analysis of iron oxidation by whole cells.

Five test tubes, each containing 3.8 ml of distilled water adjusted to pH 3.0 with H_2SO_4, were placed in a water bath at 35 C. This temperature had been established as optimal for the system. To each tube, 0.2 ml of cell suspension was added and O_2 was bubbled through the suspension via Pasteur pipettes. The rate of gas flowing through each tube was regulated by a needle valve to ensure a constant bubbling rate. At 15-sec intervals, 1 ml of a $FeSO_4$ solution containing 2.5 mg (500 μg/ml) of Fe^{2+} was added to each tube. The oxidation of iron was stopped in individual tubes by the addition of 5 ml of 2 N HCl at intervals of 2, 3.25, 6.50, and 10.75 min. The development of the yellow ferric chloride complex was estimated as above. For these assays, a reaction tube which received HCl to stop the reaction at 1 min served as the blank; the assay was not considered linear for the first minute. The optical density (OD) at 410 nm was plotted versus reaction time, and the rate of change as OD_{410} per min was obtained from a line fitted to the OD values of the reaction tubes.

The effect of whole cells and Fe^{2+} substrate concentrations on the rate of iron oxidation was followed by varying the concentration of cells or of iron in the appropriate reaction system.

The pH of the reaction mixture of the assay system was varied by using different amino acid buffers. Glycine and β-alanine were dissolved in excess dilute H_2SO_4 of known normality and back-titrated to the desired pH with KOH. The final concentrations of components in the assay system were: Fe^{2+}, 500 μg/ml; glycine or β-alanine, 0.05M; SO_4^{2-}, 0.034 M. A variety of other buffers were also tested in order to determine whether they could replace the β-alanine-SO_4^{2-} buffer.

The effect of SO_4^{2-} and Cl^- on iron oxidation was investigated. The assay system contained Fe^{2+} (200 μg/ml, as $FeSO_4 \cdot 7H_2O$); the SO_4^{2-} content was increased from 0.0036 to 0.05 M by the addition of K_2SO_4. The Cl^- levels were varied by preparing a series of assay systems in which all cations were the same and the anions consisted of various mole percentages of Cl^- and SO_4^{2-}. The test systems contained 8.94×10^{-3}M Fe^{2+}, 10^{-2}M β-alanine, and a total anion concentration of 3.79×10^{-2}M; the pH was adjusted to 3.2 with KOH.

All assay results were reproducible and all experiments were done in triplicate and with different batches of cells.

Anionic replacement of SO_4^{2-} in the iron assay system.

The assay system to test for replacement of other anions contained: 1.9×10^{-2}M Cl^-, 4.5×10^{-3}M Fe^{2+}, and 5×10^{-3}M β-alanine. The fact that Cl^- was not inhibitory at these levels and could not satisfy the anionic requirements for iron oxidation led to the use of a system containing Cl^- as the only anion to determine whether other anions could replace the SO_4^{2-} requirements for iron oxidation. The various test anions were added as the Na or K salt and were tested at 2.5×10^{-3}M, both in the presence and absence of 2.5×10^{-3}M SO_4^{2-}.

Results

The bubble tube-type of iron assay measuring the ferric chloride complex at 410 nm shows a linear increase in Fe^{3+} after a brief initial lag (Fig. 16); the lag could be due to the mixing requirement. The Fe^{3+} levels can be compared to those measured by the SCN^- assay, which also shows a linear response. However, the SCN^- assay has limitations which restrict its use for kinetic analysis; these limitations are not pertinent to this paper. The rate of iron oxidation ($\Delta OD_{410/min}$) was found to be directly proportional to the concentration of cells used in the assay over the ΔOD range of 0.02 to 0.085.

The effect of Fe^{2+} concentration (from 3.5 to 35.8 mM) on the rate of iron oxidation revealed an apparent K_m of this system to be 4.5 mM.

The standard iron assay procedure and various amino acid buffers were used to obtain more precise information concerning the effect of pH on iron oxidation by intact cells. Glycine-SO_4^{2-} buffer was used over the pH range of 1.8 to 3.6, and β-alanine-SO_4^{2-} buffer was used over the pH range of 3.2 to 4.8. The pH optimum for Fe^{2+} oxidation was broad, extending from 2.4 to 3.6 and a rapid

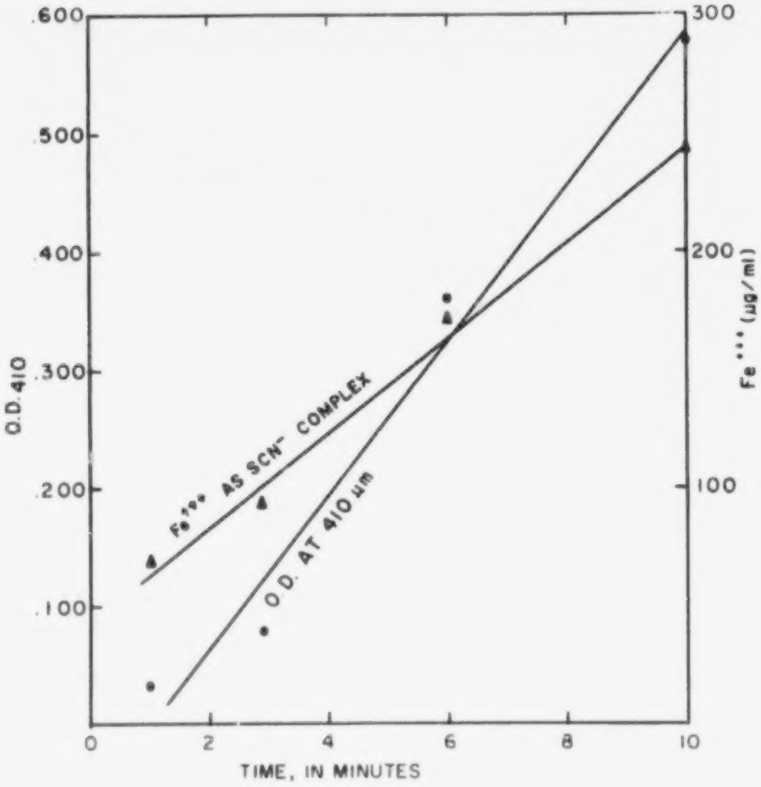


Figure 16. Bubble tube assay for iron oxidation by intact cells of *T. ferrooxidans*. Iron oxidation was followed by measuring Fe^{3+} with the SCN^- complex and by measuring the Fe^{3+}-chloride complex at 410 nm.

decrease in iron oxidation occurred above pH 3.6 and below 2.0.

The effect of pH on iron oxidation was investigated further by observing its effect on the two kinetic parameters, K_m and V_{max}. When β-alanine-sulfate buffer was used in the assay system over a pH range of 2.4 to 4.4 with the Fe^{2+} concentration varied from 3.5 to 17.9 mM for each pH, the K_m remained constant throughout most of the pH range. The change in V_{max} was more significant in terms of the pH optimum, since it decreased below pH 2.8 and above pH 3.6. The K_m determined in the presence of β-alanine-SO_4^{2-} buffer was 2.2×10^{-3} M.

Table 11 shows the effects of using various buffers in the assay system measuring Fe^{2+} oxidation by whole cells. Both formate and acetate buffer systems were inhibitory. Maleate, citrate, and malate buffers were also unsuitable, owing to inhibition of the biological oxidation or to some auto-oxidation of iron. The slight stimulation of iron oxidation observed with β-alanine may be due to additional SO_4^{2-} added to the system, but this did not occur in the case of glycine-SO_4^{2-}.

The iron assay system was also used to investigate the effects of SO_4^{2-} on iron oxidation. Addition of SO_4^{2-} to the reaction mixture, ranging from 0.0036 to 0.05 M, approximately doubled the rate of iron oxidation. Table 12 shows the effect of the addition of SO_4^{2-} containing salts. The four salts tested all caused a significant increase in iron oxidation. The low increase with $(NH_4)_2SO_4$ is probably due to a slight inhibition caused by NH_4^{2+} ions. It was shown that the addition of SO_4^{2-} increased the velocity of the reaction but did not affect the K_m.

The effects of Cl^- on the rate of iron oxidation indicated a slight stimulation of iron oxidation occurring at low Cl^- levels, but the rate decreased rapidly at high Cl^- levels; no oxidation was observed when Cl^- was the only anion present. Since these levels of Cl^- do not inhibit iron oxidation, the data indicate a specific SO_4^{2-} requirement.

The fact that Cl^- was not inhibitory and that chloride could not satisfy the anionic requirements for iron oxidation led to the use of a system containing Cl^- as the only anion, to determine whether other anions could

Table 11

Buffers tested for use in the iron oxidation assay

Buffer system	pH tested	Results
Formate-formic acid	3.2	Inhibition (70%) at $5x10^{-5}M$
Acetate-acetic acid	3.6	Inhibition (55%) at $5x10^{-2}M$
Maleate-NaOH	3.6	Slight auto-oxidation; no biological oxidations at $5 \times 10^{-2}M$
Citrate-NaOH	3.6	Slight biological oxidation; large autooxidations at $5 \times 10^{-2}M$
Malate-NaOH	3.6	Same as maleate
Glycine-SO_4^{2-}	2.1-3.7	No inhibition or stimulation; rate of oxidation the same as without buffer.
β-Alanine-SO_4^{2-}	3.2	Slight stimulation of oxidation over the control with no buffer.

Table 12

Effects of various SO_4^{2-} containing salts on the rate of iron oxidation.

SO_4^{2-} salt added	SO_4^{2-} in assay	ΔOD_{410}/min
	M	
None	0.0036	0.033
K_2SO_4	0.041	0.066
$MgSO_4$	0.041	0.060
Na_2SO_4	0.041	0.059
$(NH_4)_2SO_4$	0.041	0.046

replace SO_4^{2-}. When various anions were added to the reaction system in the presence and absence of 2.5×10^{-3}M SO_4^{2-} to test for both the inhibitory effect of anions and their ability to replace SO_4^{2-}, it was noted that both HPO_4^{2-} and $HAsO_4^{2-}$ could partially replace SO_4^{2-} (Table 13). Both of these anions stimulated iron oxidation in the presence of SO_4^{2-}, but the stimulation was no greater than that observed when the equivalent amount of addition SO_4^{2-} was added. Borate appeared to be without effect and it did not replace SO_4^{2-}. Nitrate did not replace SO_4^{2-} and was inhibitory in the presence of SO_4^{2-}; molybdate did not replace SO_4^{2-} and was strongly inhibitory. These preliminary results demonstrate that the requirement for SO_4^{2-} in iron oxidation is quite specific.

Isolation and properties of the inorganic pyrophosphatase enzyme

Materials and Methods

Organism

Thiobacillus ferrooxidans strain TM, was grown on the 9K medium as described by Silverman and Lundgren (16). Cells were grown in 16-liter carboys under forced aeration and harvested after 48-60 h using a Sharples centrifuge. The cells were suspended in 0.1 M β-alanine sulfate buffer (pH 3.6) and centrifuged at 160 x g for 10 min to remove iron; the supernatant containing cells was centrifuged at 13,000 x g for 10 min. The cells were washed twice with the same buffer and stored as a 10% (w/v) suspension until used.

Preparation of Cell-Free Extracts

Prior to breakage, washed whole cells were treated for 8-12 h with 0.5 M Tris-HCl buffer (pH 8.5) and then centrifuged at 13,000 x g for 10 min. The cell pellet was resuspended in Tris-HCl buffer and the cells were disrupted by treatment in a 10 kcycles/s Raytheon sonic oscillator for 20 min. Unbroken cells and debris were removed by centrifugation at 13,000 x g for 15 min and the supernatant was saved.

Enzyme Assays

The inorganic pyrophosphatase assay was that of Akagi and Campbell (50). The reaction mixture contained 50 μmoles

Table 13

Effect of various anions on iron oxidation in a system containing only Cl^- and in the same system containing Cl^- plus SO_4^{2-}

Anion added[a]	ΔOD_{410}/min	
	No SO_4^{2-}	2.5×10^{-3}M SO_4^{2-}
$HAsO_4^{2-}$	.005	.033
HPO_4^{2-}	.015	.035
BO_3^-	.000	.031
NO_3^-	.000	.016
MoO_4^{2-}	.000	.000
SO_4^{2-}	.030	.035[b]

[a] The assay system used to test anions other than SO_4^{2-} contained the following: 5×10^{-2}M β-alanine, 1.9×10^{-2}M Cl^-, and 4.5×10^{-2}M Fe^{2+}. The various test anions were added as the Na or K salt and were tested at 2.5×10^{-3}M both in the presence and absence of 2.5×10^{-2}M SO_4^{2-}.

[b] This assay contained a total of 5×10^{-2}M SO_4^{2-}

of Tris-HCl buffer (pH 8.0), 5 µmoles of $MgCl_2$, 5 µmoles of $Na_2P_4O_7$, enzyme, and H_2O to a final volume of 0.7 ml. All components except $Na_2P_4O_7$ were incubated at the temperature of the assay (40° C) for 5 min before the addition of substrate. After 10 min. the reaction was stopped by the addition of 1 ml of 5 N H_2SO_4 and immediate chilling of the reaction tubes. An appropriate sample was removed for inorganic phosphate determination, using a modified method (51). Unless otherwise indicated, results are expressed as units per milligram protein. A unit is 1 µmole of inorganic phosphate liberated per minute.

Protein was measured by the method of Lowry et al. (17) with crystalline bovine serum albumin as the standard.

Alkaline phosphatase was assayed by the method of Garen and Levinthal (52).

All results reported represent averages from at least three separate assays and results were reproducible using different batches of cells.

Purification Procedure

Cell-free extracts were centrifuged at 105,000 x g for 1 h in a type 65 rotor Beckman ultracentrifuge, model L2-65B. All centrifugations reported were run at 0-4° C. To the supernatant, an equal volume of 2% streptomycin solution was slowly added and the mixture stirred for 30 min in the cold. After standing for 15 min, the mixture was centrifuged at 13,000 x g for 10 min. For every milliliter of supernatant (usually 5 ml) 0.01 ml of 1 M $MgCl_2$ solution was added and the solution placed in a boiling water bath for 5 min. Denatured protein was removed by two centrifugations. Solid $(NH_4)_2SO_4$ was added slowly, with stirring, to the supernatant to 60% saturation, and the suspension stirred for 30 min in the cold and allowed to stand for an additional 15 min before centrifugation. The supernatant was treated with $(NH_4)_2SO_4$ to 90% saturation and again stirred for 30 min in the cold, allowed to stand for 15 min, and centrifuged. The precipitate was dissolved in 0.01 M Tris-HCl buffer (pH 8.0) and dialyzed overnight in the cold against the same buffer. An equal volume (45 ml) of DEAE-cellulose, equilibrated with the same buffer, was added to the dialyzed fraction and the suspensions stirred for 1 h in the cold. This volume was arbitrarily selected based upon purification experience with other enzymes (2,3). After centrifugation at 13,000 x g for 10 min, 0.1 M Tris-HCl buffer (pH 8.0) was added to the DEAE-cellulose pellet. This procedure was repeated using increasing buffer concentrations following each centrifugation. Concentrations of 0.2 M, 0.3 M, and 0.4 M were each used once, and 0.5 M Tris-HCl buffer was used twice. The supernatants from the two 0.5 M eluates were pooled and filtered to remove any residual DEAE-cellulose. This fraction was then dialyzed for 2-4 h against 40% (w/v) polyethylene glycol (Carbowax 6000) for the purpose of concentrating the enzyme.

Spheroplast Formation

Five grams of cells were washed with 0.1 M Tris-HCl buffer (pH 7.9). The cell pellet was resuspended in 50 ml of a solution containing 2.0% lipase and 0.025 M $CaCl_2$ dissolved in 0.1 M Tris-HCl buffer (pH 7.9) which was previously centrifuged at 600 x g for 10 min to remove solids. After stirring at room temperature for 2.5 h, the cells were centrifuged and the pellet resuspended in 50 ml of 0.1 M Tris-HCl buffer (pH 7.9) containing 1.0% ethylenediamine tetraacetate (sodium salt), 1.0% lysozyme, and 10.0% sucrose. The solution was stirred for 5 h at room temperature and then stored at 0-4°C overnight. After storage the cells were centrifuged and the pellet was suspended in 10.0% sucrose in 0.1 M Tris-HCl buffer (pH 7.9). For every 2 ml of the above sucrose solution, 1 ml of H_2O, 5 ml of 0.05 M $MgSO_4$ in 0.15 M NH_4Cl-NH_4OH buffer (pH 9.1), and 2 ml of 0.15 M NH_4Cl-NH_4OH buffer (pH 9.1) were added. Spheroplasts formed in a majority of the cells in several minutes as determined by phase microscopy.

Osmotic shocking of whole cells was done according to the method of Neu and Chou (53).

Results

Inorganic Pyrophosphatase Activity

Cell-free extracts possessed more inorganic pyrophosphatase activity than did intact cells which exhibited about 6% of the activity of the extracts. Activity is expressed on the basis of milligrams of protein, and it is assumed that no preferential release of enzyme occurred during cell breakage. Spheroplasting of iron-grown cells released about 11% of the enzyme activity whereas osmotic shocking of whole cells released about 7% of the activity (Table 14).

Properties of the Purified Enzyme

The purification as outlined in Table 15 shows about a 21-fold purification of the enzyme.

The activity of the purified enzyme, as determined by the assay conditions, was linear with increasing protein concentrations (0.5-6.0 μg).

The enzyme had a broad pH optimum extending from pH 7.5 to 8.5. Sodium acetate buffer (50 μmoles) was used from pH 5.5 to 6.0 and Tris-HCl buffer (50 μmoles) from pH 7.2 to 9.0. No other buffer systems were studied for their effect upon the enzyme.

The enzyme was inactive when Mg^{2+} was absent, and other cations were poor substitutes; these were Mn^{2+}, Co^{2+}, and Zn^{2+} (Table 16). The two anions (SO_4^{-2} and Cl^-) tested were without effect on the enzyme; $MgSO_4$ was as effective as $MgCl_2$.

Heat inactivation studies were done for the enzyme, which was heated at the different temperatures for 10 min. In the presence of Mg^{2+}, the enzyme was more stable to heat, and under the conditions of the assay approximately 8% of the activity remained after heating for 10 min at 100°C. In fact, results of other thermal inactivation experiments indicate that the enzyme retains 8% residual activity after heating for 1 h at 100°C. No explanation is known for this heat resistance. The possibility of the presence of more than one enzyme cannot be ruled out. Also, since the purification procedure involved a heat treatment it is not known if a heat-labile enzyme was removed. No temperature studies were made on crude cell free extracts. Blumenthal et al. (54) did report a heat-stable enzyme in cell-free extracts of gram-negative bacteria.

The inorganic pyrophosphatase was unable to hydrolyze adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), glucose 1-phosphate,

Table 14

Inorganic pyrophosphatase activity of different cell preparations

Preparation	Percent total enzyme activity
1. Cell activity	
Cell extracts	100
Intact cells	6
2. Spheroplasts	
Supernatant collected during spheroplasting	11
Spheroplast lysate*	89
3. Osmotic Shocking	
Cold water wash	7
Lysate of cells following shocking	93

* Supernatant after centrifugation of spheroplasts ruptured by sonic disintegration.

Table 15

Purification of inorganic pyrophosphatase

Fraction	Protein (mg/ml)	Activity (units*/mg protein)	Yield	Purification (fold)
I. Cell-free extract	3.80	3.99	100	1.0
II. 105,000 x g supernatant	2.00	7.25	88	1.9
III. Streptomycin	1.10	6.66	85	1.7
IV. Heat (100°C, 5 min)	0.31	20.06	60	5.2
V. Ammonium sulfate	0.12	24.08	18	6.2
VI. DEAE-cellulose	0.04	80.93	10	21.0

* A unit is defined at 1 µmole phosphate liberated per minute.

Table 16

Effect of cations on inorganic pyrophosphatase.

Cation	Percentage Activity
None	0
Mg^{2+}	100
Mn^{2+}	7
Co^{2+}	8
Zn^{2+}	11*
Ca^{2+}	0*
Ba^{2+}	0*
Fe^{2+}	0*
Cu^{2+}	0

* Incipient precipitation appeared during the assay.

Note: The reaction mixture contained 5 μmoles of each cation and 2.2 μg of protein. Mg^{2+}, Ca^{2+}, Ba^{2+}, and Fe^{2+} were supplied as chlorides; Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} were supplied as sulfates.

glucose 6-phosphate, and fructose 6-phosphate. These substrates were tested at 5 μmoles each. The enzyme did not act upon p-nitrophenyl phosphate (3 μmoles), a substrate for alkaline phosphatase; bis-p-nitrophenyl phosphate, the substrate for phosphodiasterase, was not tested.

Both ethylenediamine tetraacetate (EDTA) and potassium fluoride at a concentration of 10^{-2}M strongly inhibited (90%) inorganic pyrophosphatase. Guanidine-HCl and sodium azide (10^{-2}M) inhibited the enzyme about 25%. Two sulfhydryl-binding agents, p-chloromercuribenzoate and iodoacetamide, had no effect on the enzyme nor did increasing concentrations of phosphate (up to 10^{-2}M), the end product of the reaction (Table 17).

Effect of Pyrophosphate and Magnesium Concentrations on Enzyme Activity.

The rather specific requirement of the enzyme for Mg^{2+} prompted further study of this reaction. Figure 17 shows that except at very high Mg^{2+} concentrations, maximum enzyme activity occurred when the Mg^{2+} to pyrophosphate (PP_i) ratio was one. In the region of excess PP_i, inhibition was observed; excess Mg^{2+} was also inhibitory.

The apparent K_m of the enzyme with respect to either Mg^{2+} or PP_i was calculated as 1.6×10^{-3}M from a Lineweaver-Burk (14) plot. To obtain this plot the initial reaction velocities for low substrate concentrations of PP_i and a Mg^{2+}/PP_i ratio of one were used.

Discussion

An understanding of structure-function relationships in the cell envelope and how they relate to chemolithotrophy was the purpose of the first part of this section. The lipopolysaccharide outer layer of iron grown *T. ferrooxidans* isolated by hot phenol-water extraction was similar to other gram negative LPS molecules as observed in the negatively stained preparations. The isolated LPS were ribbon-like as well as forming distinct sheets of LPS containing many visible holes. Preliminary chemical analysis indicate that the basic sugar content of the LPS was similar to *E. coli* and *Salmonella* (49) LPS. The isolated LPS molecule had a molecular weight in excess of a million. The LPS also contained a rather large amount

Table 17

The effect of metabolic inhibitors on inorganic pyrophosphatase

Inhibitor (10^{-2}M)	Percentage activity
None	100
EDTA	4
Potassium fluoride	10
Guanidine-HCl	79
Sodium azide	73
p-Chloromercuribenzoate	100
Iodoacetamide	100
Orthophosphate	100

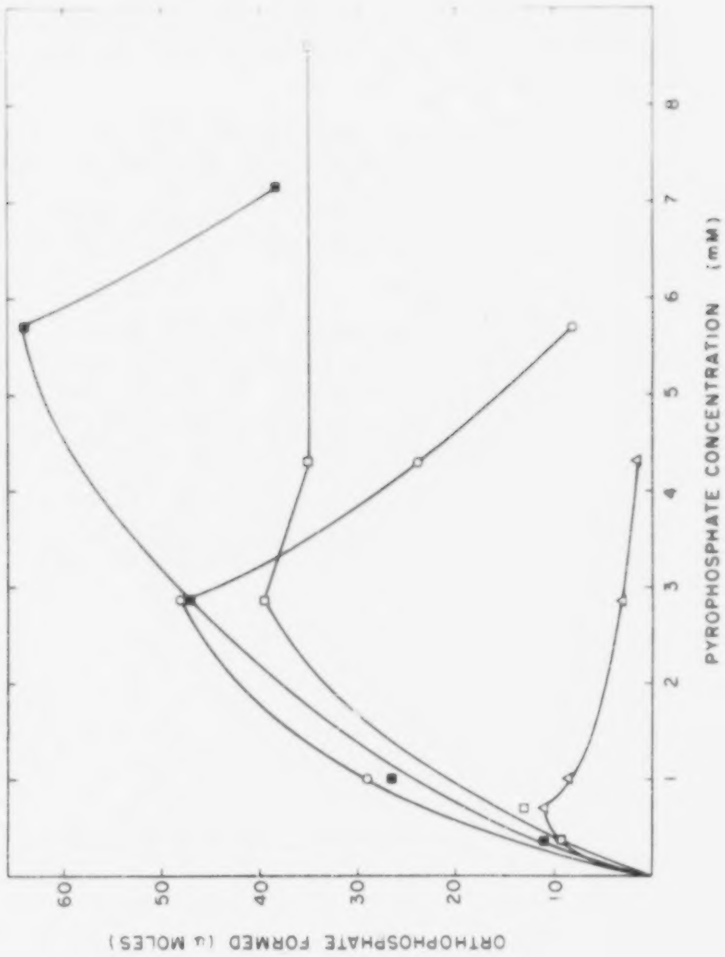


Figure 17. Effect of inorganic pyrophosphate concentration on inorganic pyrophosphatase activity with varying Mg^{2+} concentrations. The enzyme assay was run under standard conditions and 2.2 μg of protein were used. (Δ) 0.715 mM Mg^{2+}, (O) 2.86 mM Mg^{2+}, (■) 5.72 mM Mg^{2+}, (□) 8.58 mM Mg^{2+}.

of ferric iron. The presence of the ferric iron may substantiate the role of the outer layer of cell in ferrous iron oxidation.

The whole cell kinetic assay technique described in the second part of this section allows one to study the rates of iron oxidation by iron-oxidizing bacteria. The advantages of the assay procedure are its simplicity and rapidity, as compared to the conventional Warburg respirometer.

Using the new method, it was found that SO_4^{2-} was a requirement for iron oxidation. This had been previously reported (55). This requirement may relate to the fact that SO_4^{2-} is produced by the cell during sulfur oxidation. It appears, from these data, that the SO_4^{2-} is involved in the binding of ferrous iron to the cell for subsequent oxidation. The sulfate anion were partially replaced by HPO_4^{2-} and to a lesser extent by $HAsO_4^{2-}$. Formate and molybdate inhibited the iron oxidation reaction, under the new assay conditions.

The inorganic pyrophosphatase studied was found to be located in the cells cytoplasm. The partially purified enzyme required Mg^{2+} for activity and could not be replaced by Mn^{2+}, Zn^{2+}, or Co^{2+}. It is generally believed that the Mg^{2+} forms a complex with the pyrophosphate molecule ($MgPPi^{2-}$) and it is this complex on which the enzyme acts. The K_m for the enzyme (1.6 mM) is in general agreement with inorganic pyrophosphatases isolated from other microbes.

One aspect of this enzyme which appears repeatedly in the isolation and characterization of other *T. ferrooxidans* enzymes, is the alkaline pH optimum. All the enzymes isolated to date from *T. ferrooxidans* (2,3) have alkaline pH optima, while the pH of the aqueous environment in which the cells exist is so low (pH 2.5-3.5). This observation further emphasizes the role of the outer cell envelope in maintaining a pH balance between the acid environment and the cells cytoplasm.

SECTION VII

STUDIES CONCERNING THE METABOLISM OF *THIOBACILLUS FERROOXIDANS* GROWN ON HETEROTROPHIC SUBSTRATES

Introduction

The acidophilic iron-oxidizing bacteria include the group of organisms previously referred to as *Ferrobacillus ferrooxidans*, but now recognized as *Thiobacillus ferrooxidans* (56, 28). A chemolithotroph, *T. ferrooxidans* obtains energy for the reduction of carbon dioxide from the oxidation of reduced iron and sulfur compounds. Recent studies, however, have shown that not only this organism, but other autotrophs, can use organic compounds such as sugars, amino acids and various metabolic intermediates for energy (57). Lundgren, *et al* (58) have shown that when autotrophically-grown *T. ferrooxidans* is transferred to a medium containing iron plus glucose, cells preferentially utilize ferrous iron and when iron is exhausted, the cells oxidize glucose as an energy source upon transfer into a glucose-salts medium.

Some strains of iron-oxidizing bacteria are unable to adapt to glucose, and it has been suggested that this trait may be considered as a basis for species differentiation (57). Interestingly, glucose has been shown to inhibit iron and elemental sulfur oxidation in manometric experiments using resting cells prepared from cultures grown on either inorganic substrate (59). Concomitant with the inhibition of substrate oxidation, the autotrophic fixation of carbon dioxide is drastically reduced in the presence of high concentrations of glucose. It has recently been found that glucose inhibits CO_2 fixation from 40-66 percent when sulfide, thiosulfate, tetrathionate, dithionate or sulfite served as the source of reduced sulfur (60). Other studies (J. H. Tuttle and P. R. Dugan, Bacteriol. Proc., p. 64, 1969) have shown several organic acids to inhibit both iron and sulfur oxidation.

This investigation was undertaken to study the effects of organic metabolites, particularly glucose, on the growth and metabolism of *T. ferrooxidans* in an attempt to understand metabolic changes induced by the oxidation and subsequent catabolism of this sugar. Further, glucose-6-phosphate dehydrogenase, a key enzyme of glucose dissimilation was studied to ascertain its possible role in the control of heterotrophic growth by autotrophic bacteria.

Materials and Methods

Organism and Growth Conditions

T. ferrooxidans strain TM was grown in the 9K medium as previously described (16).

Growth on Iron-Glucose Media

When it was desired to grow *T. ferrooxidans* on the 9K medium, supplemented with glucose (9 KG medium), the 9K salts and ferrous sulfate solution (at pH 2.5) were autoclaved separately. Upon cooling, the two solutions were combined and filter-sterilized glucose was added at 0.5% final concentration. The final pH was 2.7. 10 ml of a culture of iron-grown *T. ferrooxidans* (5×10^9 cells) was aseptically added to the 9 KG medium (100 ml total volume) in a 250-ml Erlenmeyer flask. The flask was shaken on a reciprocal shaker at 30 C for 60 h, at which time all the ferrous iron was oxidized. The contents of this flask served as an inoculum for 500 ml of 9 KG medium, contained in a Fernbach flask. 9K-fructose-grown, 9K-sucrose-grown, and 9K-glutamate-grown cells were cultured in a similar manner, with the exception that the appropriate sugar or amino acid substrate was substituted for glucose. Cells were harvested as described for iron-grown cells.

Growth on Glucose

T. ferrooxidans was readily cultured in a glucose 9K salts media. This was done by transferring 10 ml of a 9 KG culture into 90 ml of a glucose-salts medium free from substrate amounts of $FeSO_4$. The glucose was filter-sterilized at high concentrations (10%) and added to sterile 9K-salts solution (pH 2.5) at a final concentration of 0.5%. Cells have been continuously transferred, and maintained on the glucose-salts medium for over a year, with bimonthly transfers to fresh medium. Large masses of glucose-grown cells were used for preparing crude enzyme extracts; cells were grown using a 10% inoculum from a fresh starter culture and inoculated into 500 ml of the glucose-salts medium contained in 2800-ml Fernbach flasks. Flasks were shaken at 30 C on a 3-tiered New Brunswick rotary shaker set at 150 cycles/min.

Cells were grown on several organic media, merely by replacing the appropriate organic substrate for glucose.

In all cases where media contained an organic energy source, contamination was vigorously monitored by streaking cultures on a battery of complex media, including nutrient agar, Saboraud's agar, thioglycollate and nutrient broth, adjusted to different pH's. Plates (or tubes) were incubated at various temperatures for at least ten days. In no case, was contamination observed. Microscopic examination of the cultures was also used to monitor purity.

Growth Rates

Growth rates for cultures grown on sugars or other organic substrates were determined using 250-ml Nephalo flasks fitted with side arms (Bellco Glass Inc., Vineland, New Jersey). The flasks contained 20 ml of medium and were shaken at 150 cycles/min at 30 C; turbidity was followed spectrophotometrically at 550 nm in a Bausch and Lomb Spectronic 20 colorimeter and absorbance plotted as a function of culture time. The generation time was calculated from the exponential portion of the growth curve and is defined as the time required for absorbance at 550 nm to double. The specific growth rate constant, k, was determined from the expression

$$k = \frac{\ln 2}{t_2 - t_1}$$

where $t_2 - t_1$ equals the time interval required for cell doubling (61).

Colorimetric Determination of Iron

Iron was determined by the procedure described by Suzuki and Silver (20) using 2,2'-dipyridyl.

Whole Cell Iron Oxidation Assay

The procedure for the colorimetric assay used for kinetic studies of iron oxidation by whole cells as described earlier in this report was used to measure iron oxidation.

Glucose Assay

Glucose was assayed using the Glucostat enzyme reagent (Worthington Biochemical Corp., Freehold, N. J.). All dilutions of standards and samples were made in 10 mM potassium phosphate, pH 7.0. 9.0 ml of the Glucostat reagent were placed in colorimeter tubes to which was added 1 ml of the sample. After exactly

10 min at room temperature, one drop of 4 N HCl was added to stop the reaction and stabilize the color. The tubes were allowed to stand for 5 min and then read at 400 nm.

Radiorespirometric Studies

The radiorespirometric experiments were performed as described by Wang et al (62), as modified by Perry and Evans (63). The experiments were done in the laboratory of Dr. J. J. Perry, Department of Microbiology, North Carolina State University, Raleigh, N. C.

Preparation of Cell-free Extracts by Sonic Oscillation

Harvested cells of *T. ferrooxidans* were immediately suspended in the appropriate buffer at 4 C, washed three times and suspended in the same buffer overnight at 4 C. This eliminated the necessity of the treatment of the cells with ion-exchange resins (2) to facilitate rupture of *T. ferrooxidans*. The cells were centrifuged at 35,000 x *g* for 10 min, suspended as a 20% (u/v) suspension in buffer and disrupted in a water-cooled 10 kc/sec Raytheon sonic oscillator for 15 min. Residual whole cells and debris were removed by centrifugation at 15,000 x *g* for 20 min and the resulting supernatant was used as the crude cell-free extract. Preparation of crude cell-free extracts for ribulose diphosphate (RuDP) carboxylase assay was carried out in this manner; the buffer, at pH 7.9, contained 50 mM Tris-HCl, 12 mM Tris-HCl, 12 mM 2-mercaptoethanal, 50 mM $NaHCO_3$ and 15 mM EDTA. Extracts to be used for assay of glucose catabolic enzymes were prepared from cells suspended in 50 mM Tris-HCl (pH 7.9). Tricarboxylic (TCA) cycle enzymes were assayed using cell-free extracts cells previously harvested as described above and immediately suspended in a potassium phosphate (50 mM)-glutathione (1 mM) buffer at pH 7.4. Cell pellets were twice washed with this buffer. The presence of glutathione (GSH) or dithiothreitol (1 mM) in the buffer was essential for maximum enzymatic activity. The cells were suspended to a 20% w/v suspension in the phosphate-GSH buffer, placed in a standard Raytheon cup and N_2 gas bubbled through the cell suspension for 10 min. The cells were disrupted by sonic energy for 15 min. After the removal of whole cells and debris, the supernatant was separated and used for assay of the TCA cycle enzymes, as well as for NADH oxidase assays.

Cell extracts were also prepared from cells harvested at different times during growth in the 9 KG medium, in this instance 2 ml of a 20% (w/v) cell suspension were treated using a Branson sonifier, equipped with a mini-probe

(Branson Instruments, Inc., Plainview, New York). The cell suspension was chilled in an ice bath and intermittent bursts of energy were used, amounting to a total of 5 min.

Protein Determination

Protein was determined coloimetrically by the method of Lowry et al (17) using crystalline bovine serum albumin as the standard. The spectrophotometric method of Warburg and Christian, as described by Layne (64) was also used for estimating protein.

Assay for RuDP Carboxylase

Ribulose 1,5-diphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39 was determined in a 2-step coupled assay essentially as described by Stukus and DiCicco (1970). Step 1 results in the formation of 3-phosphoglyceric acid. The reaction mixture, in a total volume of 2.0 ml contained: Tris-HCl (pH 7.9), 50 mM, $MgSO_4$, 6 mM; GSH, 6 mM; $NaHCO_3$, 40 mM; RuDP, 1 mM, extract and water to 2.0 ml. The reaction was initiated at 37 C with extract and run for 5 min. The reaction was stopped by placing reaction tubes in a boiling water bath for 3 min. After cooling in an ice bath, denatured protein was removed by centrifugation and the supernatant drawn off and used. Step 2: The 3-phosphoglyceric acid formed in step 1 as a result of RuDP carboxylase activity was determined in a coupled assay containing 3-phosphoglyceraldehyde dehydrogenase (230 μg) and 3-phosphoglyceric acid kinase (2.2 μg). The reaction mixture also contained: Tris-HCl (pH 7.9), 50 mM; ATP, 0.67 mM; $MgSO_4$, 6.67 mM; L-cysteine (pH 7.0), 6.67 mM; NADH (pH 7.0), 0.13 mM; one ml of step 1 supernatant and water to 3.0 ml. A unit of activity is defined as the number of μmoles of 3-phosphoglyceric acid formed in 1 min, as measured by following the oxidation of NADH at 340 nm. Activity is expressed as units/mg of protein calculated from the linear portion of a curve plotting enzyme activity vs protein concentration. A Coleman #124 recording spectrophotometer, equipped with absorbance expansion from 0 to 0.1 absorbance, was used for the assay.

Assay for Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate; $NADP^+$ oxidoreductase, EC 1.1.1.49) was assayed spectrophotometrically at 340 nm in 3 ml cuvettes with a 1 cm light path, by following the rate of appearance of either NADPH or NADH. The reaction mixture contained: Tris-HCl (pH 7.9), 15 mM; MgCl$_2$, 6.67 mM; glucose-6-phosphate, 0.83 mM; NADP$^+$, 0.33 mM or NAD$^+$ (pH 7.0), 3.33 mM; cell-free extract, suitably diluted in 50 mM Tris (pH 7.9) and water to 3.0 ml. The reaction, in the case of crude extracts, was initiated with either NADP$^+$ or NAD$^+$. Enzyme was used to initiate the reaction during kinetic experiments. When NADH was included in the cuvette, the reduction of NADP$^+$ was measured at 366 nm. A Coleman 124 recording spectrophotometer was used routinely in all assays. For kinetic studies, a Gilford Model 240 spectrophotometer coupled to a Leeds and Northrup Speedomax G recorder and equipped with a temperature controlled sample holder, was used at 25 C.

Assay for 6-Phosphogluconate Dehydrogenase

6-phosphoglyconate dehydrogenase (6-phospho-D-glyconate: NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.44) was assayed using the same assay as for glucose-6-phosphate dehydrogenase except that 2.67 mM 6-phosphoglyconate was used in place of glucose-6-phosphate. The potassium salt of 6-phosphoglyconate was prepared from the barium salt according to the procedure of Horecker and Smyrniotis (65).

Assay for Fructose-1, 6-diphosphate Aldolase

Fructose diphosphate aldolase (fructose-1, 6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC 4. (1.2.1.3) was measured spectrophotometrically using a coupled assay containing triosephosphate isomerase and α-glycerophosphate dehydrogenase; the rate of NADH oxidation was followed at 340 nm, and the activity of the enzyme calculated from the initial rate of NADH oxidation. The reaction mixture contained: Tris-HCl (pH 7.5), 0.1 M; fructose-1, 6-diphosphate, 5 mM; 0.02 ml α-glycerophosphate dehydrogenase/triosephosphate isomerase (10 mg/ml); NADH (pH 7.0), 0.25 mM; cell extract and water to 3.0 ml. The reaction was initiated by adding cell extract.

Assay for the Entner-Doudoroff Enzymes

The Entner-Doudoroff enzymes, 6-phosphogluconate dehydrase (6-phosphogluconate dehydratase, EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) were assayed by determining the amount of pyruvate formed from 6-phosphoglyconate, using the procedure of Keele, Hamilton and Elkan (66). 1 unit of enzyme activity results in the formation of 1 μmole of pyruvate from 6-phosphogluconate. Activity is expressed in units per mg of protein.

Assay for Isocitrate Dehydrogenase

Isocitrate dehydrogenase (threo-D_s-isocitrate: NAD^+ oxidoreductase (decarboxylating) EC 1.1.1.41) and (threo-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) was assayed spectrophotometrically at 340 nm following the rate of appearance of NADH or NADPH. The reaction mixture contained Tris-HCl (pH 7.9), 10 mM; $MnCl_2$, 0.33 mM; DL-isocitrate, 0.17 mM; NAD^+, 3.33 mM or $NADP^+$, 0.33 mM; extract and water to 3.0 ml.

Assay for Aconitase

Aconitase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) was assayed according to the procedure of Anfinsen (67), based on the spectrophotometric determination at 240 nm of cis-aconitic acid.

Assay for Succinic Dehydrogenase

Succinate dehydrogenase [succinate: (acceptor) oxidoreductase, EC 1.3.99.1] was assayed spectrophotometrically at 600 nm following the reduction of 2,6-dichlorophenol-indophenol (DCPIP), mediated by phenazine methosulfate (PMS), according to the procedure of Arrigoni and Singer (68).

Assay for α-Ketoglutarate dehydrogenase was assayed spectrophotometrically at 600 nm by following DCPIP reduction as described by Watson and Dworkin (69).

Assay for Fumerase

Fumerase (fumerate hydratase, EC 4.2.1.2) was assayed spectrophotometrically at 240 nm according to the procedure of Racker (70).

Assay for NADH, Oxidase

NADH oxidase [NADH: (acceptor) oxidoreductase, EC 1.6.99] was assayed spectrophotometrically at 340 nm by following the oxidation of NADH. The reaction mixture contained NADH, 0.25 mM; phosphate buffer (pH 7.4), 66.7 mM; cell-free extract and water to 3.0 ml. The reaction was initiated with extract.

For all assays controls were routinely performed and consisted of the reaction mixture minus the specific substrate, or minus the cell-free extract. All enzymes were inactivated by boiling for 5 min. In all cases, except where noted, enzyme was expressed as units per mg of protein. One unit is the amount of enzyme needed to oxidize or reduce one μmole of substrate per min. Assays were always repeated three times and results were reproducible with different batches of cell-free extract.

In kinetic experiments with glucose-6-phosphate dehydrogenase, velocities are given as nmoles of NADPH or NADH formed per min. In Lineweaver-Burk plots, the reciprocal of velocity is given as $1/\Delta\ OD_{340}$/min.

Purification of Glucose-6-Phosphate Dehydrogenase

All enzyme purification steps reported were conducted at 0-4 C. All centrifugations were performed at 35,000 g in a Sorvall RC2-B centrifuge. The sequential treatments were:

> Step 1. The crude cell-free extract of *T. ferrooxidans*, prepared as described, was frozen in a dry ice-ethanol bath of -20 C. The extract was stored at this temperature until needed. At this time, the frozen extract was thawed and then centrifuged for 20 min; the resulting precipitate was discarded. The remaining supernatant was then centrifuged at 105,000 x g in a Beckman model L-2 preparative ultracentrifuge. The pellet was again discarded, while the clear yellow-orange supernatant was used for enzyme purification.
>
> Step 2. To the 105,000 x g supernatant, solid ammonium sulfate was added slowly with stirring over a period of 15 min until the concentration reached 35% saturation. The solution was centrifuged for 15 min and the pellet discarded. The resulting supernatant was brought to 50% saturation with ammonium sulfate with constant stirring. The mixture was centrifuged for 20 min. The resulting precipitate contained nearly all the enzyme activity.
>
> Step 3. The 50% ammonium sulfate precipitate was suspended in a minimum amount of 0.05 M Tris-HCl buffer, pH 7.9, and applied to a Sephadex G-200 column (2.5 x 30 cm) equilibrated with 0.05 M Tris-HCl (pH 7.9) and eluted with the same buffer, collecting 10 ml fractions. Enzyme of high specific activity was usually found in a

single tube, and this material was used in all future purification steps.

Step 4. The eluate from the Sephadex column was fractionated with ammonium sulfate, first to 35% saturation and then to 50% saturation as before. The precipitate was collected by centrifugation and resuspended in a minimum volume of 0.05 M Tris-HCl, pH 7.9. This material was either chromatographed on a small Sephadex G-200 column (1.5 x 20 cm) in the case of enzyme from glucose-grown cells, or was dialyzed against two 1-liter changes of 0.05 M Tris-HCl buffer, pH 7.9, in the case of enzyme from iron-glucose grown cells (9 KG cells).

Step 5. The enzyme from the glucose-grown cells was eluted off the small G-200 column by 0.05 M Tris-HCl, pH 7.9 in 4 ml fractions. Usually one of these fractions contained the highest specific activity and this fraction was used for all subsequent experiments. Further purification was unsuccessful.

The dialyzed preparation from iron-glucose-grown cells was further purified by negative $Ca_3(PO_4)_2$ gel absorption: $Ca_3(PO_4)_2$ gel, purchased commercially, was added at a ratio of 3 mg of calcium phosphate to every 2 mg of protein. This mixture was stirred for 15 min slowly and then centrifuged for 20 min. The supernatant was then treated again with additional calcium phosphate (3 mg to every 2 mg of protein). The resulting supernatant, following centrifugation, was used in all cases as the enzyme from iron-glucose-grown cells.

Disc Gel Electrophoresis

The standard disc gel electrophoretic procedure of Davis (18) was used. When gels were to be stained for protein, gels were first fixed for 20 min in 20% trichloroacetic acid (TCA), stained for 40 min with Coomassie blue (1% aqueous solution diluted 1:20 with 20% TCA), and destained with 10% TCA.

Stains to detect enzyme activity were performed in 6% gels by placing gels in small tubes protected from the light. The tubes contained the assay components with the addition of phenazine methosulfate (PMS) and nitrobluetetrazoleum (NBT) at 0.056 mg/ml and 0.90 mg/ml respectively. The gels were incubated for 15 min at room temperature after which they were stored in 10% TCA. Gels placed in tubes lacking substrate were not stained. The position of the enzyme in the gel was determined by scanning stained gels at 600 nm in a Gilford spectrophotometer, with a linear transport attachment. The relative mobility of the stained enzyme (R_m) was defined as the ratio of the migration (cm) of the enzyme from the origin to the migration (cm) of the dye front from the origin.

In all cases, enzyme was layered on the lower gel in 5% sucrose and electrophoresed at pH 9.3, using the anionic system described in the Buchler Instruments pamphlet "Instructions for the Poly-analyst," Buchler Instruments, Inc., Fort Lee, N.J. The current was maintained at 3 ma per gel.

Molecular Weight Determinations

Molecular weights were determined using gels of different acrylamide concentration, according to the method of Hedrick and Smith (19). The gels were stained for activity and R_m's were plotted against gel concentration; the slope of this line was determined using a computer program written by Mr. Amnon Liphshitz (Dept. of Biology, Syracuse University) and fitted to a standard curve of several proteins of known molecular weight.

Chemicals and Reagents

The chemicals and reagents used in this study were mainly procured from commercial sources, and were of the highest purity available. The sources of some of these are listed below.

Sigma Chemical Company: ADP, AMP, 3', 5' - AMP, ATP, DCPIP, DTT, GSH, NAD^+, NADH, $NADP^+$, NADPH, NBT, PMS, Tris, glucose-6-phosphate, 6-phosphogluconate, ribulose 1,5-diphosphate.

New England Nuclear Corporation: glucose-1-^{14}C, glucose-2-^{14}C, glucose-3-^{14}C, glucose-3,4-^{14}C, glucose-6-^{14}C.

Packard Instrument Corporation: PPO and POPOP.

Fisher Scientific Company: 2,2'-dipyridyl, ferrous sulfate, D-glucose, D-fructose, sucrose, potassium gluconate, sodium glutamate, sodium acetate, sodium succinate, sodium pyruvate.

Nutritional Biochemicals Corporation: enzyme grade ammonium sulfate, calcium phosphate gel.

Canalco: acrylamide, glycine, ammonium persulfate N, N, N', N'-tetramethylethylenediamine, N, N'methylenebisacrylamide, bromphenol blue, Coomassie blue.

All reagents and buffers were prepared in glass distilled water.

Results

Effects of Organic Metabolites on Autotrophic Mechanisms

Utilization of Glucose by Iron-grown Cells

Iron-grown cells required at least two transfers in a medium containing both iron and glucose (9 KG medium) before glucose could be utilized as a sole source of energy. Figure 18A and 18B show the relationship of iron and glucose utilization by iron-grown cells. Heavy inocula (5 g wet weight) were used in these experiments insuring that all the ferrous iron would be rapidly oxidized and leaving glucose as the sole energy source. Ferrous iron was oxidized to ferric iron by 2-3 hr, whereas the glucose level remained constant for over 62 hr. When an inoculum of these cells was transferred to fresh 9 KG medium, cells quickly oxidized the iron and then, after a lag, oxidized the glucose (Fig. 18B). This lag, prior to glucose oxidation, had been noted previously (58), and was reproducible in the present author's hands. The increase in the level of glucose-6-phosphate dehydrogenase (G6PD), a key enzyme for the dissimilation of glucose, is also shown. The utilization of glucose by the cells resulted in the accumulation of poly-B-hydroxybutyric acid (PHB).

Iron Oxidation by Resting Cells Cultured on Iron and Organic Supplements.

T. ferrooxidans was cultured on ferrous iron plus an organic supplement, and the substrate's effect on the iron oxidizing capacity of thiobacilli tested using washed resting cells. Resting cells were prepared from cultures harvested after exhaustion of ferrous iron. The rate of iron oxidation by cells previously grown in the various supplemented media varied according to the organic substrate present. Cells grown in the iron

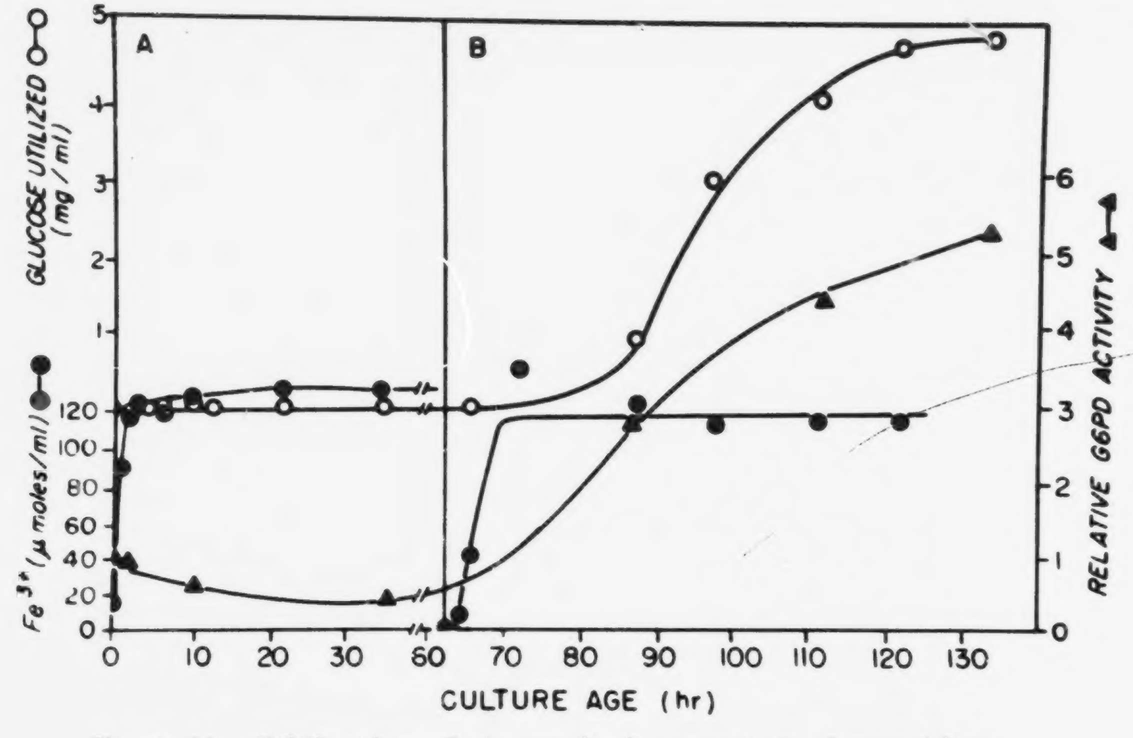


Figure 18. Utilization of glucose by iron-grown T. ferrooxidans. 5 g of iron-grown cells (wet weight), suspended in the salts of the 9K medium, were added to 500 ml of the iron-glucose supplemented medium (pH 2.7) contained in a 2-liter Erlenmeyer flask. The flask was incubated at 30 C and shaken at 150 cycles/min. At zero time and intervals thereafter, samples were removed for ferric iron, glucose, and enzyme assays. After 62.5 hr of incubation, 50 ml of medium containing about 0.5 g of cells were placed in a flask containing fresh medium and the shaking continued. Samples were removed aseptically and analyzed as before (Fig. 18B). Glucose-6-phosphate dehydrogenase (G6PD) activity (units/mg of protein) is expressed relative to the resting cell inoculum.

glucose medium lost 80% of their iron oxidizing ability (Table 18), whereas iron-fructose-grown cells oxidized iron at a rate nearly comparable to cells grown solely on ferrous iron. These results demonstrate that once organic carbon is oxidized by the cells, the autotrophic energy generating system is affected, however, the degree depends upon what particular organic metabolite is supplied.

Ribulose Diphosphate Carboxylase Levels

The levels of ribulose diphosphate (RuDP) carboxylase in cell free extracts prepared from cells grown in the various supplemented media are shown in Table 19. Extracts from iron-fructose and iron-glutamate grown cells possess about 70% of the carboxylase activity of autotrophically grown cells. However, both sucrose and glucose almost completely repress the synthesis of this enzyme.

Relationship Between Iron Oxidation and RuDP Carboxylase Activity

The relationship between iron oxidation, as expressed in resting cells previously grown in a 9 KG medium, and carboxylase activity in extracts of these cells is illustrated in Figure 19. The greatest amount of carboxylase activity (3.5 h) is noted just after completion of iron oxidation, thereafter enzyme activity diminishes to the level of the cell inoculum (t_o). Washed resting cells prepared from cells previously grown in the 9 KG medium for 1-6 h and then assayed for iron oxidation and enzyme activity exhibited the highest iron oxidation rate at 1 hr; at this time Fe^{3+} ion is actively accumulating in the growth medium. Maximum iron oxidation rates precede peak RuDP carboxylase activity. These results indicate that the two processes, iron oxidation and CO_2 fixation, so necessary for autotrophic existence, are related and that maximum iron oxidation must precede the maximum expression of carboxylase. The presence of glucose in the medium does not affect the RuDP carboxylase activity or iron oxidation rates, for at the early hours of growth only ferrous iron is oxidized.

Heterotrophic Growth of T. ferrooxidans

T. ferrooxidans was cultured on glucose as the sole energy source. Glucose disappeared from the medium by 50 h and the cell doubling time was 4.5 h. Glucose-grown cells examined under the phase microscope were

Table 18

Effect of organic supplement(s) on iron oxidation by resting cells of *T. ferrooxidans* previously grown in the iron-supplemented medium[a]

Growth substrate(s)	ΔOD_{410}/min (10^2)[b]	Cell dry weight (mg)	ΔOD/min/mg (10^2)	% of control
9K (iron)	2.80	0.40	7.00	100.0
9K-fructose	1.60	0.30	5.41	77.3
9K-sucrose	0.95	0.38	2.53	36.1
9K-glutamate	0.80	0.38	2.13	30.4
9K-glucose	0.56	0.36	1.57	22.4

[a] In each case, cells were harvested after 60 hr of growth, washed in β-alanine-SO_4 buffer, pH 3.6, and suspended in this buffer.

[b] Iron oxidation was determined using the kinetic assay as described by Schnaitman *et al* (71).

Table 19

Ribulose 1,5-diphosphate carboxylase levels in extracts of *T. ferrooxidans* grown in the iron-supplemented media[a]

Growth substrate[b]	Units/mg (10^{-3})	Autotrophic level (%)
Iron	9.88	100.0
Iron + glucose (60 hr)	1.55	15.7
Iron + glucose	0.54	5.5
Iron + sucrose	0.51	5.2
Iron + fructose	6.62	67.0
Iron + glutamate	7.17	72.6

[a] All cells were harvested after 66 hr of growth except for one instance (60 hr), labeled above.

[b] Organic supplements were added to a final concentration of 0.5%.

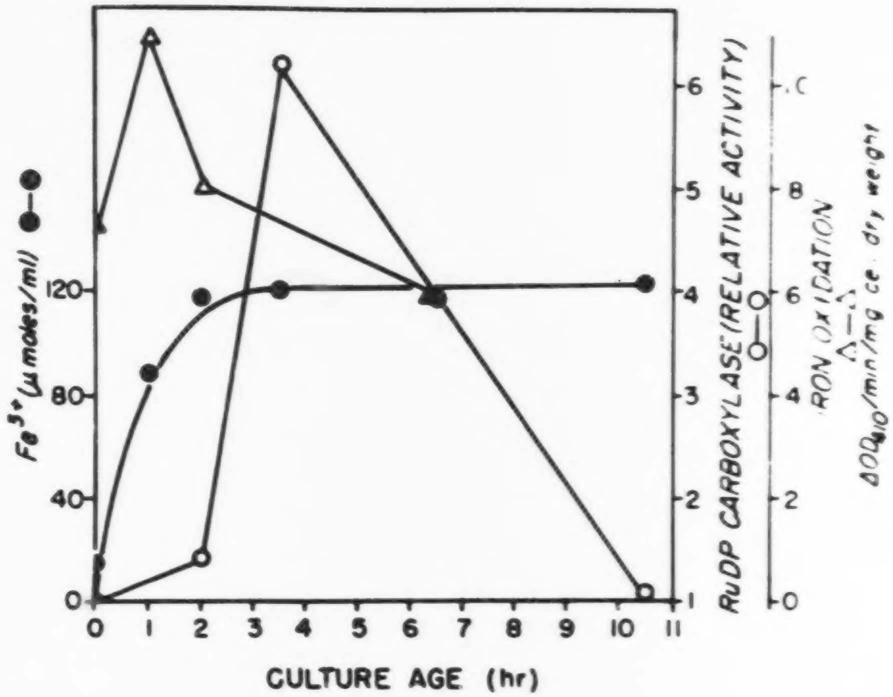


Figure 19. Relationship of the rate of iron oxidation by resting cells to RuDP carboxylase activity. Cells were grown in 500 ml of the 9KG medium (pH 2.5) using 5 g of iron-grown cells as an inoculum. Fe^{3+} was determined in culture supernatants at the times indicated. For iron oxidation and RuDP carboxylase assays, 9 KG-grown cells were harvested and washed as previously described. Iron oxidation by resting cells was assayed spectrophotometrically at 410 nm. RuDP carboxylase (units/mg of protein) is expressed relative to the level of the enzyme found in the cell inoculum (zero time).

found in pairs and contained large amounts of the refractile storage product PHB. Glucose-grown cells were readily transferred to defined media containing other sugars or organic substrates in place of glucose (57). Growth rates of the organism grown on five different types of substrates are compared in Table 20. Fructose appeared to be the preferred substrate with a generation time of 3.4 hr; all other substrates tested gave generation times greater than glucose-grown cells. Cells grown heterotrophically on any of the aforementioned substrates gradually regained their potential for autotrophic growth when transferred back to the 9K medium. Glucose-grown cells showed no detectable iron oxidation ability; cells grown on the other substrates were not tested.

No absolute requirement for vitamins or growth factors was noted for cells grown in the organic media. Glycerol, acetate, pyruvate, and succinate (at a concentration of 0.5%) did not support growth. No other organic substrates were tested.

Heterotrophic Metabolism of T. ferrooxidans

As shown previously, iron-grown cells, once adapted to glucose, possessed glucose-6-phosphate dehydrogenase, the enzyme which catalyzes the initial reaction in both the pentose-phosphate shunt and the Entner-Doudoroff pathways. These results suggested that in order to metabolize glucose, drastic changes in intermediary metabolism must take place. Studies were therefore initiated to determine the enzymatic basis for the change from an autotrophic existence to a heterotrophic one. Radiorespirometric experiments, using ^{14}C-specifically labeled glucose, were also performed to help determine the pathways) through which glucose is metabolized.

Enzymes of Glucose Catabolism

Enzymes functioning in the Embden-Meyerhof, Entner-Doudoroff and pentose-phosphate pathways were assayed using crude cell-free extracts of *T. ferrooxidans* grown autotrophically, mixotrophically (iron + glucose), and heterotrophically. The results (Table 21) show that during autotrophic growth (iron-grown cells), the levels of the glucose catabolic enzymes were low, whereas, as previously shown, the capacity for iron oxidation and CO_2 fixation was high. This low level of the glucose enzymes in autotrophically-grown cells indicates that these enzymes

Table 20

Growth of *T. ferrooxidans* on organic substrates

Growth substrate (0.5%)	Generation time (hr)	Growth rate constant k (hr^{-1})
Glucose	4.5	.154
Sucrose	6.6	.105
Fructose	3.4	.204
Glutamate	7.4	.094
Gluconate	7.0	.099

Table 21

Effect of growth substrate on enzymes involved in glucose metabolism by _T. ferrooxidans_[a]

Enzyme	Activity (units/mg x 10^3)		
	Iron-grown cells	Iron-glucose-grown cells	Glucose-grown cells
Glucose-6-phosphate dehydrogenase ($NADP^+$)	1.29	52.70	76.67
Glucose-6-phosphate dehydrogenase (NAD^+)	1.55	62.00	90.20
6-phosphogluconate dehydrogenase ($NADP^+$)	3.70	N.D.[b]	N.D.
6-phosphogluconate dehydrogenase (NAD^+)	1.23	1.02	1.59
6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase[c]	0.40	12.4	15.9
Fructose-1,6-diphosphate aldolase	1.29	0.47	0.88

[a] In each case, cells were taken from the late log phase of growth.

[b] N.D. = not detected under the conditions used.

[c] 1 unit of activity results in the formation of 1 nmole per min. of pyruvate from 6-phosphogluconate.

function in biosynthesis, and do not appear to be directly involved in energy metabolism (72). However, when glucose serves as an energy source, as with iron-supplemented medium or in the glucose-salts medium, glucose-6-phosphate dehydrogenase (both NADP$^+$-and NAD$^+$-linked) was induced about 50 times above the autotrophic level. The presence of an inhibitor to glucose-6-phosphate dehydrogenase in crude cell extracts was ruled out, for adding this extract to cell-free extracts of *E. coli* had no effect; *E. coli K12* is known to have a high level of glucose-6-phosphate dehydrogenase. Nor was inhibition observed when extracts from iron-grown cells were added to partially purified glucose-6-phosphate dehydrogenase obtained from glucose-grown *T. ferrooxidans*. Either NADP$^+$ or NAD$^+$ served as coenzyme for the glucose-6-phosphate dehydrogenase from *T. ferrooxidans*. The activity of 6-phosphogluconate dehydrogenase was low or undetectable in all extracts tested. However, the enzymes of the Entner-Doudoroff pathway (6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase) in cell extracts from iron-supplemented and glucose-grown cells showed a 30-40 fold stimulation over extracts prepared from iron-grown cells.

The assay for the enzymes of the Entner-Doudoroff pathway was based on the conversion of 6-phosphogluconate to pyruvate. The enzymatic formation of pyruvate was confirmed by comparing the absorption spectra of the dinitrophenylhydrozones of standard pyruvate and the enzymatically generated compound. Further, the $\frac{OD_{490}}{OD_{540}}$ ratio of both products (1.12 for the pyruvate standard, 1.07 for the sample) are compatible and correspond to values obtained by Smith and Gunsalus (73). In each case, there is an optical density difference of .015 from 490 to 540 nm. In the assay ferrous ions are needed for maximum activity (Table 22). It is also apparent that the reaction will not proceed without 6-phosphogluconate or extract. Boiling for 5 min destroys over 85% of the activity. Reduced glutathione, however, does not appear to be essential. The reaction is linear with protein concentration, up to about 3 mg of protein.

As a further control, the formation of pyruvate with time using 6-phosphogluconate as substrate was compared, using extracts of *T. ferrooxidans* grown on iron (9K),

Table 22

Requirements for the Entner-Doudoroff enzyme assay

Deletions	Pyruvate formed[a] (nmoles)	% of control
None	340	100
Gluconate-6-phosphate	0	0
Cell-free Extract	0	0
GSH	340	100
Fe^{++}	224	65.9
None (enzyme boiled for 5 min)	46	13.5

[a] represents the amount of pyruvate (nmoles) formed in 30 min.

iron-supplemented medium (9 KG) and *Pseudomonas aeruginosa*, an organism known to degrade glucose by way of the Entner-Doudoroff pathway (74).

The high levels of glucose-6-phosphate dehydrogenase and Entner-Doudoroff enzymes were not observed when other sugars or carbon sources were used for energy substrates (Table 23). Glucose-grown cells were readily transferred to several different organic substrates which supported good growth (Shafia and Wilkinson, 1969). However, these cells showed altered activities for both glucose-6-phosphate dehydrogenase and Entner-Doudoroff enzymes. Cells grown on sucrose or fructose apparently do not use the Entner-Doudoroff pathway. Exactly how these various sugars are metabolized by *T. ferrooxidans* remains to be determined.

Enzymes of the TCA Cycle

Five representative enzymes of the TCA cycle, plus NADH oxidase, were assayed in cell-extracts prepared from iron-grown (autotrophic), iron-glucose-grown (mixotrophic), and glucose-grown cells. Enzyme activities were generally lower in extracts from iron-grown cells (Table 24); succinic dehydrogenase, α-ketoglutarate dehydrogenase and NADH oxidase were not detected in these extracts. NADP$^+$-linked isocitrate dehydrogenase, fumarase and aconitase were very low, whereas NAD$^+$-linked isocitrate dehydrogenase levels were high. The presence of glucose (iron-glucose grown cells) activates succinic dehydrogenase, α-ketoglutarate dehydrogenase and NADH oxidase and greatly stimulates NADP$^+$-linked isocitrate dehydrogenase, fumarase and aconitase. NAD$^+$-linked isocitrate dehydrogenase levels remained unchanged in the presence of glucose. This same enzyme pattern was expressed in extracts prepared from cells grown solely on glucose; the TCA cycle enzymes were operative and NADH oxidase activity was readily apparent.

Radiorespirometric studies

To better understand which pathway(s) was involved in glucose dissimilation, cells grown on the iron-glucose combination medium and the glucose-salts medium were placed in radiorespirometer vessels and CO$_2$ formation followed using ^{14}C-specifically labeled glucose. The observed labeling patterns indicate the nature of the pathways involved (62). Results of these studies with iron-glucose-grown cells are given in Figure 20. A very active decarboxylation

Table 23

Effect of organic substrate on the activity of glucose-6-phosphate dehydrogenase and the Entner-Doudoroff enzymes

Growth substrate[a]	Relative enzyme activity[b]	
	Glucose-6-phosphate dehydrogenase	Entner-Doudoroff enzymes[c]
Glucose	1.00	1.00
Sucrose	1.08	0.06
Fructose	0.41	0.08
Glutamate	0.20	0.41
Gluconate	0.25	0.46

[a] All organic substrates were added at 0.5% final concentration; cells were taken from the late log phase of growth.

[b] Enzyme activity is compared to the levels found in glucose cells.

[c] 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase.

Table 24

Activity of TCA cycle enzymes and NADH oxidase in cell extracts of _T_. _ferrooxidans_ grown on various media[a]

Enzyme	Activity (units/mg x 10^{-3})		
	Iron-grown cells	Iron-glucose-grown cells	Glucose-grown cells
Isocitrate dehydrogenase ($NADP^+$)	0.65	55.4	61.5
Isocitrate dehydrogenase (NAD^+)	33.3	34.7	27.3
Aconitase	2.30	1050	1690
Succinic dehydrogenase	N.D.[b]	54.3	179
α-ketoglutarate dehydrogenase	N.D.	30.4	9.70
Fumarase	33.0	982	456
NADH oxidase	N.D.	17.0	30.4

[a] In each case, cells were taken at the late log phase of growth.

[b] N.D. = not detected under the conditions used.

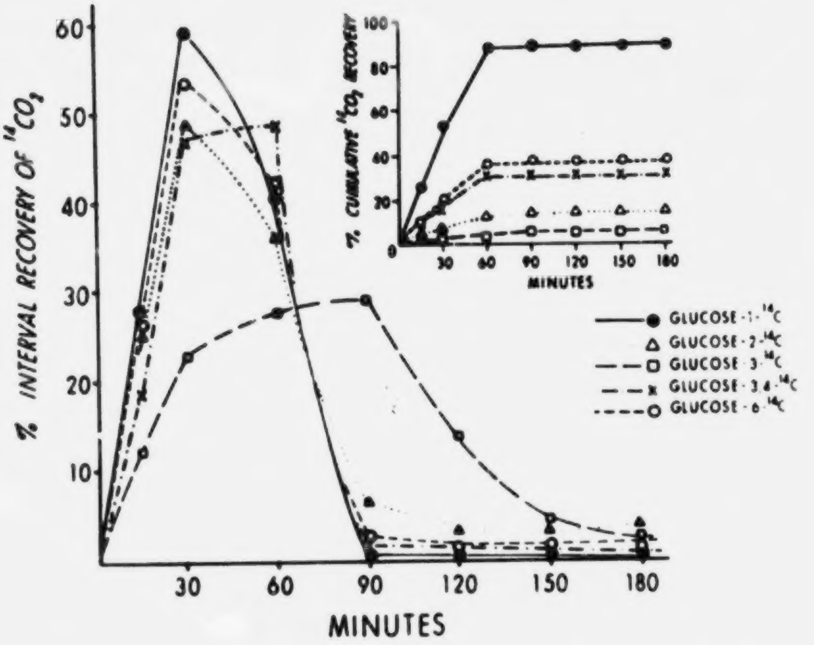


Figure 20. Radiorespirometric pattern for the utilization of specific ^{14}C-labeled glucose by iron-glucose-grown cells of T. ferrooxidans. 19.2 mg of cells (dry weight) were suspended in the basal salts solution making up the iron containing medium (pH 2.5).

occurred at the C-1 position, and a peak respiration rate was reached within 30 min. The rate of CO_2 evolution of C-4 labeled glucose (determined by extrapolation) was higher than from C-3 labeled glucose; C-3 was respired less rapidly, with peak evolution of respiratory CO_2 occurring after 90 min. The enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase splits glucose between carbons 3 and 4; thus carbons 1 and 4 will appear as the carboxyl groups of the resulting pyruvate molecules. If the Emden-Meyerhof pathway were operable, CO_2 derived from C-3 and C-4 of glucose would appear before CO_2 derived from C-1. However, in T. ferrooxidans grown in the iron-glucose medium, it appears that much of the glucose was dissimilated by the Entner-Doudoroff pathway, with a strong contribution by the pentose shunt mechanism. The high CO_2 production from C-6 was unexpected and suggests that the triose phosphate produced from glucose dissimilation via the Entner-Doudoroff scheme, and comprising carbon atoms 4, 5 and 6, may be metabolized in an unorthodox manner. Analysis of the cumulative $^{14}CO_2$ recovery from glucose (Table 25) allows one to calculate the relative contribution of the Entner-Doudoroff and pentose phosphate pathways. For iron-glucose-grown cells, 37.1% of the glucose is channeled through the Entner-Doudoroff pathway, while 62.9% of the administered glucose is catabolized by this organism via the pentose-phosphate shunt.

A similar radioactive inventory was performed on glucose-grown cells (Table 26). In this medium the Entner-Doudoroff pathway contributes 77.5% to glucose catabolism, with the pentose-phosphate shunt responsible for 22.5% of the glucose dissimilated.

If glucose is metabolized through the Entner-Doudoroff pathway, the triose phosphate formed must be diverted into hexose phosphate. The hexose-phosphate is then degraded by the pentose cycle (75). If this explanation were correct, $^{14}CO_2$ recovered would be rich from carbon 1 and fairly rich from carbon 6. The percent $^{14}CO_2$ recovered substantiate that carbon 1 is indeed evolved at a high rate, with CO_2 evolved from carbon 6 at substantial, but lower, levels. This form of glucose metabolism would also necessitate that carbon 2 be retained as acetate, possibly leading to the synthesis of PHB, known to accumulate in this organism.

Table 25

Utilization of ^{14}C-labeled glucose by iron-glucose-grown _T_. _ferrooxidans_[a]

Substrate	Radioactive distribution[b]			Total ^{14}C recovery
	Respiratory CO_2	Supernatant	Cells	
	%	%	%	%
Glucose-1-^{14}C	89.0	1.5	5.6	96.1
Glucose-2-^{14}C	15.2	8.7	57.5	81.4
Glucose-3-^{14}C	6.1	3.8	81.0	90.9
Glucose-3,4-^{14}C	31.7	1.2	60.9	93.8
Glucose-6-^{14}C	37.3	4.0	46.4	87.7

[a] 19.2 mg (dry wt) cells were added to 20 ml of the salts of the 9K medium.

[b] Counts collected at the end of the experiment.

Table 26

Utilization of ^{14}C-labeled glucose by glucose-grown T. ferrooxidans[a]

Substrate	Radioactivity distribution[b]			Total ^{14}C recovery
	Respiratory CO_2	Supernatant	Cells	
	%	%	%	%
Glucose-1-^{14}C	42.4	54.9	1.1	98.4
Glucose-2-^{14}C	20.2	51.7	12.1	84.0
Glucose-3,4-^{14}C	18.7	75.8	3.3	98.8
Glucose-6-^{14}C	17.5	50.8	10.1	78.4

[a] 53.2 mg (dry wt) cells were added to 20 ml of the salts of the 9K medium.

[b] Counts collected at the end of the experiment.

Glucose-6-phosphate Dehydrogenase

Purification and stability of T. ferrooxidans glucose-6-phosphate-dehydrogenase

Glucose-6-phosphate dehydrogenase, a branch point enzyme leading to both the pentose-phosphate shunt and the Entner-Doudoroff pathway, was partially purified from both iron-glucose and glucose-grown cells. The purification procedure involved preparative ultracentrifugation to remove membrane fragments, ammonium sulfate fractionation, Sephadex chromatography and negative calcium phosphate adsorption. Glucose-6-phosphate dehydrogenase from iron-glucose grown *T. ferrooxidans* was eluted from the Sephadex G-200 column just after the major protein peak. This elution profile was obtained also from preparations from glucose-grown cells. Table 27 gives a summary of the purification of glucose-6-phosphate dehydrogenase from cell-free extracts of iron-glucose grown cells. The enzyme, after treatment with calcium phosphate gel, was purified about 90 fold over the crude cell-free extract with a recovery of about 15%. Disc gel electrophoresis of this enzyme preparation stained for protein showed one large band along with several minor bands.

The enzyme from glucose-grown *T. ferrooxidans* was purified about 150 fold with about a 4% recovery (Table 28). This preparation also gave 1 large major band on polyacrylamide gels stained for protein along with 6 or 7 minor electrophoretic components.

For both purified preparations, neither NADH nor NADPH oxidase nor 6-phosphogluconate dehydrogenase, enzymes capable of interfering with the assay of glucose-6-phosphate dehydrogenase, were detected. The enzyme from iron-glucose or glucose-grown cells was stored frozen at -20 C in small samples. Upon thawing, 10-15% of the activity was lost. Activity was gradually lost at room temperature (50% within 10 h).

Enzyme activity of the purified preparation was linear with protein from 0 to 20 μg of protein using either the $NADP^+$ or NAD^+-linked reaction.

Table 27

Purification of glucose-6-phosphate dehydrogenase from *T. ferrooxidans*

grown in an iron-glucose medium

Fraction	Total Units	Total protein (mg)	$\frac{\text{Units}}{\text{mg}}$	Recovery (%)
Crude extract	51.3	1036.0	0.05	100.0
1st Ammonium sulfate	36.5	86.0	0.42	71.0
G-200 eluate	19.5	18.0	0.67	38.1
2nd Ammonium sulfate	18.9	8.1	2.33	36.8
Calcium phosphate gel	7.5	1.7	4.34	14.5

Table 28

Purification of glucose-6-phosphate dehydrogenase from glucose-grown

T. ferrooxidans

Fraction	Total units	Total protein (mg)	Units/mg	Recovery (%)
Crude extract	407.3	5087.3	0.08	100.0
Ammonium sulfate	239.6	315.0	0.76	58.9
1st G-200 eluate	74.0	44.1	1.68	18.2
2nd G-200 eluate	15.4	1.3	11.9	3.8

Pyridine Nucleotide Specificity of Glucose-6-phosphate Dehydrogenase.

T. ferrooxidans glucose-6-phosphate dehydrogenase, at each stage of the purification, was capable of utilizing either $NADP^+$ or NAD^+ as coenzyme (Table 29). The ratio of the $NADP^+$-linked activity with respect to the NAD^+-linked reaction at each purification stage was 0.8-0.9, indicating that there was a single enzyme capable of both $NADP^+$ or NAD^+ reduction. The confirmation for the dual nucleotide specificity of *T. ferrooxidans* glucose-6-phosphate dehydrogenase was shown by activity staining on polyacrylamide gels. When the enzyme from iron-glucose grown cells was electrophoresed and gels stained for enzyme activity, similar scanning profiles were obtained in the presence of both coenzymes (Table 30). In each case, a large enzyme peak (peak a), along with a smaller peak (peak b) was found. The enzyme isolated from glucose-grown cells and stained in the presence of both $NADP^+$ and NAD^+, exhibited similar profiles. The R_m values of each enzyme preparation, stained in the presence of both $NADP^+$ and NAD^+ are compared. The data indicate that for both the iron-glucose and glucose-cell preparation, a single enzyme catalyzes both $NADP^+$-and NAD^+-linked activity. Moreover, the R_m's for the enzyme from both iron-glucose and glucose-grown-cells are the same. Thus, glucose-6-phosphate dehydrogenase obtained from growth in a mixothrophic (iron-glucose) medium shows the same electrophoretic properties as the enzyme isolated from a purely heterotrophic (glucose) environment. The partially purified enzyme from each source exhibits identical $NADP^+$:NAD^+ activity ratios. The same coenzyme activity ratios were found for the enzyme from iron-grown cells. However, in the case of extracts from cells grown autotrophically, electrophoretic activity stains were not successful due to the extremely low levels of enzyme activity.

Molecular Weight of Glucose-6-phosphate Dehydrogenase

Straight lines were obtained when per cent gel concentration was plotted against log R_m for enzyme isolated from iron-glucose grown cells and from glucose-grown cells. The slopes of the lines obtained were essentially the same for each preparation. When these slopes were fitted to a standard curve relating the known molecular weight

Table 29

Comparison of reaction velocity of glucose-6-phosphate dehydrogenase with NAD^+ and $NADP^+$ during purification of the enzyme from *T. ferrooxidans*[a]

Fraction[b]	Velocity[c]		Ratio
	$NADP^+$	NAD^+	$NADP^+/NAD^+$
Crude extract	.099	.109	0.91
105,000 x g Supernatant	.092	.103	0.89
1st 35% Ammonium sulfate supernatant	.086	.097	0.89
2nd 50% Ammonium sulfate precipitate	.029	.032	0.91
2nd G-200 eluate[d]	.058	.068	0.85

[a] Cells were grown in the glucose-salts medium.

[b] 0.1 ml of suitably diluted enzyme from each fraction was used.

[c] Velocity is expressed as ΔOD_{340}/min.

[d] Enzyme from this step was frozen, diluted and then assayed.

Table 30

Electrophoretic migration of purified glucose-6-phosphate dehydrogenase from *T. ferrooxidans*

Enzyme Source	Coenzyme	Peak	R_m
Glucose cells	NAD^+	a	0.416
		b	0.287
Glucose cells	$NADP^+$	a	0.422
		b	0.297
Iron-glucose cells	NAD^+	a	0.417
		b	0.298
Iron-glucose cells	$NADP^+$	a	0.408
		b	0.292

of several proteins to the slopes obtained for log R_m vs % gel concentration plots, the molecular weight of *T. ferrooxidans* glucose-6-phosphate dehydrogenase was determined to be 110,000.

Effect of pH on Glucose-6-phosphate Dehydrogenase Activity

When the enzyme was assayed with $NADP^+$ at different pH's of Tris buffer, a relatively sharp optimum was obtained at pH 7.8-7.9. Enzyme activity with NAD^+ as coenzyme differed in that no activity was obtained at pH's below 7.5; a relatively broad pH optimum was observed from pH 8.0-8.5. This pattern emerged regardless of the source of enzyme.

Activation by Magnesium Ions

Glucose-6-phosphate dehydrogenase purified from several microbial sources has been reported to be activated by Mg^{++} ions (61, 76, 77), yet this cation is not an absolute requirement for activity. Recently, it has been reported that the *E. coli* enzyme retained 20% of its activity in the absence of divalent cations (77). The enzyme from *T. ferrooxidans* retains 50% of its activity in the absence of Mg^{++}. The effect of Mg^{++} on the velocity of the reaction catalyzed by *T. ferrooxidans* glucose-6-phosphate dehydrogenase was tested. From Lineweaver-Burk plots, an apparent K_m (Mg^{++}) of 4.0 mM was obtained. Mn^{++} cations were effective at low concentrations (0.27 mM) in activating the enzyme; however, at higher concentrations, this effect was markedly diminished. At optimum concentrations of both cations, Mg^{++} was substantially more effective. Fe^{++} was not tested since it is autooxidized at pH's above 4.0. No other cation was tested.

Apparent Michaelis Constants

The Michaelis constants for $NADP^+$, NAD^+, and G-6-P were obtained by conventional procedures for the enzyme isolated and purified from iron-glucose-grown cells. For glucose-6-phosphate dehydrogenase isolated from glucose-grown cells, Michaelis constants for each of the above substrates were obtained similarly. In all cases, hyperbolic curves were obtained from velocity versus substrate concentration plots. There was no apparent sigmoidicity. The apparent K_m's for the substrates for the enzyme from both iron-glucose and glucose-grown cells were comparable (Table 31) and indicate along with the electrophoretic data that the enzyme is the same regardless of whether the cell is grown under mixotrophic or heterotrophic conditions.

Effect of NADH

NADH has been reported to inhibit glucose-6-phosphate dehydrogenase isolated from *E. coli* in an allosteric manner (77). However, glucose-6-phosphate dehydrogenase isolated from the chemolithotroph *T. ferrooxidans* does not exhibit a sigmoidal inhibition curve with NADH, indicating that NADH is not an allosteric effector in this case. Other workers (78) have previously shown that the enzyme from the facultative chemolithotrophy *Hydrogenomonas H-16* (grown on fructose) is noncompetitively inhibited by NADH. Thus, from results with at least two different autotrophic species, NADH does not appear to be an allosteric effector for glucose-6-phosphate dehydrogenase. In addition, Olive et al (79) have recently shown that homogeneous *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase is not allosterically affected by NADH.

Effect of ATP

ATP has been implicated as an allosteric effector with respect to glucose-6-phosphate concentration for glucose-6-phosphate dehydrogenases isolated and purified from a number of microbial sources (61, 78). The effect of ATP concentration on the velocity of glucose-6-phosphate dehydrogenase isolated from *T. ferrooxidans* was assayed with NADP$^+$. The kinetics for this inhibition by ATP at various concentrations was done. In no instance did ATP affect the hyperbolic nature of the velocity versus substrate concentration curve. From double reciprocal plots it appears that ATP inhibits in a nearly competitive manner when NADP$^+$ is used as coenzyme. Dixon plots confirm the inhibition by ATP and reflect the apparent sigmoidicity found a K_i for ATP of approximately 3 mM is obtained at 50% inhibition. Hill plots of these data give slopes of 0.9-1.2. ATP does not appreciably change the slope and the values for *n* obtained (~ 1), indicate that G-6-P binds to one site on the enzyme.

Table 31

Comparison of apparent Michaelis constants for partially purified *T. ferrooxidans* glucose-6-phosphate dehydrogenase

Enzyme source	Apparent K_m (M)		
	$NADP^+$	NAD^+	G-6-P
Iron-glucose-grown cells	1.02×10^{-5}	6.58×10^{-4}	5.18×10^{-5}
Glucose-grown cells	2.43×10^{-5}	2.08×10^{-3}	5.39×10^{-5}

ATP exhibits a profound effect on the NAD$^+$-linked reaction. The kinetics of the ATP inhibition of the NAD$^+$-linked reaction with respect to varying G-6-P concentration were tested. Again, no deviation from normal Michaelis-Menten kinetics was observed. Double reciprocal plots indicate competitive inhibition when NAD$^+$ is used as coenzyme. Dixon plots confirm the competitive nature of this inhibition. From these plots, a K_i of about 0.3 mM for ATP was obtained, 10 times less than the value obtained with NADP$^+$ used as coenzyme. Thus, ATP is 10 times more inhibitory to the NAD$^+$-linked reaction. Hill plots give slopes of 0.9-1.1 in the absence or presence of ATP for the NAD$^+$-reaction.

Effect of Other Adenine Nucleotides.

The inhibition by ATP is not shared by other adenine nucleotides (ADP, AMP, cyclic-3', 5' AMP) (Table 32). Only in the presence of ATP was substantial inhibition (80-90%) noted.

Discussion

Effect of Organic Metabolites on Autotrophic Mechanisms

The results of this study document the ability of T. ferrooxidans to grow on glucose as the sole source of energy. Shafia and Wilkinson (57) had confirmed an earlier observation of such growth by this organism (58). Implicit in each of these studies, however, was the observation that autotrophically grown T. ferrooxidans failed to grow in the glucose medium unless the cells were first grown in a ferrous sulfate medium containing 0.5% glucose. After transfer, once again, in the iron-glucose media, the progeny of this second culture were fully adapted to glucose. In the present study, large quantities (5 g) of autotrophically grown T. ferrooxidans, when incubated in the iron-glucose medium, failed to oxidize the glucose; the ferrous sulfate, however, was completely oxidized within 3 h. When these cells were subsequently transferred to fresh iron-glucose media, glucose was fully oxidized within 60 h. Noteworthy in this experiment, is the fact that the cells prefer iron to glucose; glucose is utilized some time (10 h) after the completion of iron oxidation. It may be that the further addition of the inorganic substrate, iron, is needed to provide energy for the initial transport of glucose across the cell membrane. This appears to be the case with several of the chemolithotrophic bacteria

Table 32

Effect of adenine nucleotides on *T. ferrooxidans*[a] glucose-6-phosphate dehydrogenase[b]

Nucleotide[c]	Velocity	Per Cent Activity
---	6.77	100
ATP	1.45	21.4
ADP	5.00	73.9
AMP	5.65	83.5
3', 5'-AMP	5.65	83.5
ATP + AMP	1.37	20.2
ATP + 3', 5'-AMP	0.73	10.8
ATP + ADP	1.05	15.5

[a] Enzyme obtained from glucose-grown cells.

[b] The reaction mixture in a total volume of 1.0 ml contained: Tris-HCl (pH 7.9), 15 mM; G-6-P, 1 mM; NADP$^+$, 0.35 mM; enzyme, 2 μg, and adenine nucleotide.

[c] All adenine nucleotides were at 3 mM concentration.

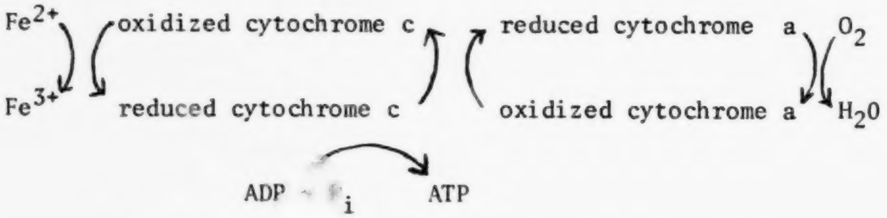
examined to date; e.g., the addition of ammonium chloride roughly doubled the incorporation of several amino acids by resting cells of Nitrosomonas europaea (80). The observed lag period before glucose is oxidized by T. ferrooxidans may also represent a period when the cell is synthesizing "transport specific" enzymes such as Enzyme II of the phosphotransferase (PTS) system of Kundig, et al (81). No experimental evidence is available to support the transferase suggestion. Alternatively (or concurrently), the cell may be synthesizing necessary inducible enzymes for glucose dissimilation during the lag period after iron oxidation. One enzyme involved in the catabolism of glucose is glucose-6-phosphate dehydrogenase, an enzyme whose activity roughly parallels the utilization of glucose in the iron-glucose medium. Poly-β-hydroxybutyrate (PHB) synthesis also parallels the utilization of glucose by T. ferrooxidans.

Once adapted to glucose from the iron-glucose medium, T. ferrooxidans was able to grow on glucose as its sole energy source. However, glucose assimilation in a thiosulfate-glucose medium by T. intermedius ceases with exhaustion of thiosulfate (82). Thus, T. ferrooxidans appears to resemble T. novellus and various Hydrogenomonas species which grow on simple organic compounds. On glucose, T. ferrooxidans showed a generation time of 4.5 h, as opposed to about 8-10 h on iron (56) and about 22 h on sulfur. Glucose-grown cells differed significantly in morphology, as compared to autotrophically grown T. ferrooxidans (83). Glucose cells usually appeared in pairs or short chains and appeared to possess many PHB storage granules, causing the cells to bulge (83).

T. ferrooxidans was also capable of growth on a variety of organic substrates (57). In this investigation, fructose appeared to give the highest growth rate. The growth stimulation by yeast extract is concentration dependent and suggests a cofactor requirement for maximum growth, possibly p-aminobenzoic acid (PABA), as suggested by Shafia and Wilkinson (57). However, no studies have been made to establish this.

T. ferrooxidans failed to grow on the organic acids acetate, pyruvate or succinate. If the hypothesis of Rao and Berger (84) is correct, this would be expected. Thus, at the low pH required for growth, these acids would enter the cell in an undissociated form, then dissociate and permit the further passive entry of acids. As acid accumulates in the cell, there would be a concomitant decrease in internal pH with a subsequent inhibition of growth.

In contrast to the studies of Shafia and Wilkinson (57) with strain BCU-4, the adaptation to glucose has a profound effect on the cells' iron oxidation ability. This effect is also found when other organic compounds were added to the iron containing media. The degree of iron oxidation was dependent on the particular energy source; cells taken from an iron-glucose medium lost almost 80% of the ability to oxidize iron, while cells taken from an iron-fructose medium retained almost 80% of the cells iron oxidation potential. In each case cells were removed from the medium after the $FeSO_4$ had been oxidized. There is support for the belief that repression of the autotrophic machinery is likely, for assays were done with washed resting cells in the absence of any organic supplement. Thus, it would appear that a product of heterotrophic metabolism is responsible for the repression of the autotrophic iron oxidation mechanism. Currently, there is only one pathway thought to be responsible for iron oxidation, that is, through the cytochrome chain (25, 85, 86)



Iron oxidation involves cytochromes c and a, and the enzymes iron-cytochrome c reductase and cytochrome oxidase, in that order. Any repressor of iron oxidation would necessarily have to act at the level of energy generation, presumably at the cytochrome chain. An analogous situation has been described in Thiobacillus novellus, where fermentable substrates such as glucose, glycerol, lactate, ribose and pyrivate repressed most of the enzymes involved in sulfur oxidation (87). However, the degradation of glucose cannot give rise to any of the intermediates of sulfur oxidation which might then cause a type of feedback inhibition. Nevertheless, in T. ferrooxidans (59) and in T. novellus, glucose exerts a profound influence on the sulfur oxidizing systems. Thus, the effector responsible for the repression would necessarily be a product of heterotrophic metabolism. This effector would also be a product of the oxidation of iron or sulfur compounds if this system adheres to the definition of Magasanik (88) which states that the synthesis of certain enzymes is regulated by some intracellular products of the enzymes concerned. The only common by-products of both inorganic iron or sulfur metabolism and heterotrophic metabolism are ATP and NADH. Presumably the level of these energy-rich compounds would vary depending upon the ability of the cell to degrade the organic compound in question. According to Lejohn, Van Caseele and Lees (87), the cell is probably adjusting its energy imbalance by reducing the production of excess energy through some form of inhibition. In the case of *T. ferrooxidans*, iron oxidation and the resulting formation of ATP would fall into this category.

In order for autotrophic organisms to exist, there must be some mechanism for assimilating carbon dioxide directly into cell substance. The means by which this occurs is through the reductive pentose-phosphate cycle or Calvin-Benson pathway (89). The key reaction in this scheme is catalyzed by ribulose diphosphate (RuDP) carboxylase. The reaction catalyzed by this protein results in the fixation of 1 molecule of carbon dioxide to a molecule of ribulose 1,5-diphosphate with a concomitant dismutation to yield two molecules of phosphoglyceric acid. In *T. ferrooxidans*, a repression of RuDP carboxylase activity was found in cell-free extracts prepared from iron-glucose, iron-sucrose, iron-fructose, and iron-glutamate grown cells. This has also been shown with several facultatively autotrophic species, London and Rittenberg (82) reported repression of thiosulfate oxidation in *T. intermedius* as well as a decreased level of RuDP carboxylase prepared from cells propagated in a medium containing thiosulfate and an organic supplement. McFadden and Tu (90) also noted a similar repression of enzyme activity in *Hydrogenomonas facilus*.

There is a relationship between inorganic iron oxidation and RuDP carboxylase activity. It was found that maximum iron oxidation, during growth and by resting cells, precedes the expression of maximum RuDP carboxylase activity. This is to be expected since the oxidation of ferrous iron provides energy (ATP) which is then utilized in the reduction of CO_2 via the Calvin cycle. The reduction of CO_2 is a completely endergonic process, requiring large amounts of ATP and NADH. Any reduction in the amount of ATP supplied to the system will result in diminished CO_2 fixation and lower levels of RuDP carboxylase.

Heterotrophic Metabolism of *T. ferrooxidans*.

Growth on glucose affords the cell an alternate mechanism for energy generation through a form of heterotrophic metabolism. From the present results, based upon enzymatic studies and ^{14}C-labeling data, the Embden-Meyerhof pathway does not appear to be the major route of glucose dissimilation in *T. ferrooxidans*.

Instead, glucose is dissimilated through the Entner-Doudoroff pathway involving glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase. The aforementioned activities are enhanced several fold during glucose catabolism. The Embden-Meyerhof pathway probably plays a minor role in providing carbon skeletions for biosynthesis. Thus, this chemoautotroph is similar to *Thiobacillus intermedius* (72), *Hydrogenomonas eutropha* (91) and *Hydrogenomonas H-16* (92) in using the Entner-Doudoroff scheme for glucose dissimilation.

Pyruvate and 3-phosphoglyceraldehyde are the products of glucose breakdown through the Entner-Doudoroff pathway. For energy to be derived from this degradation, these products must be channeled through the TCA cycle. In *T. ferrooxidans* grown on glucose, an increase in activity of TCA cycle enzymes was noted. It has been suggested that the TCA cycle merely serves a biosynthetic function in chemoautotrophs (93), since there is an apparent absence of α-ketoglutarate dehydrogenase and low levels of succinic dehydrogenase. A lack of NADH oxidase was also reported and given as the reason for the inability of the complete TCA cycle to function (93). Several authors, however, have found NADH oxidase to be present in extracts from several chemolithotrophs (72, 94, 95). From results of these studies, *T. ferrooxidans* appears to have an incomplete TCA cycle when grown autotrophically, as well as an apparent absence of NADH oxidase. However, when grown heterotrophically (on glucose) the TCA cycle is functional and the cells exhibit an increase in $NADP^+$-linked isocitrate dehydrogenase, aconitase, fumarase, succinic dehydrogenase, and α-keto-glutarate dehydrogenase. NADH oxidase was also readily detected in glucose-grown cells. Evidence is thus accumulating supporting the idea that the TCA cycle in chemolithotrophic bacteria is incomplete and functions as a biosynthetic mechanism when autotrophic conditions exist. The presence or absence of NADH oxidase in autotrophically grown cells probably reflects a function of the regulation of its activity (96).

The presence of two isocitrate dehydrogenases (NAD^+ - and $NADP^+$-linked) may well have important regulatory functions for this organism. It is known that the NAD^+- enzyme is activated by AMP. Thus, if the NAD^+-and $NADP^+$-enzymes compete for the same pool of isocitrate, any excessive energy drain, such as the Calvin cycle, will favor the NAD^+ enzyme. This would occur since the relative concentrations of ATP would decrease, while that of AMP would increase. Thus, electrons would be channeled through the electron transport chain rather than toward biosynthesis. By contrast, under conditions where ATP accumulates in high concentrations, i.e., as the result of glucose catabolism, the $NADP^+$-enzyme would be stimulated. In addition, citrate would accumulate (due to the equilibrium of the aconitase reaction), stimulating fatty acid synthesis by activation of acetyl coenzyme A carboxylase. Under these conditions acetate could be stored in the form of PHB.

Thiobacillus intermedius is similar to *T. ferrooxidans* in that an $NADP^+$-specific isocitrate dehydrogenase is induced by glucose, while the activity of the NAD^+-enzyme remains constant (72).

Labeling experiments with ^{14}C-specifically labeled glucose show an early release of C-1 and C-4 labeled CO_2, indicating that the Entner-Doudoroff pathway is operating. Respiratory CO_2 from C-3 is evolved at least 1 h later and at substantially lower levels. Thus, the Embden-Meyerhof pathway cannot be of importance in energy generation, for if this pathway were operating, the preferential rate of evolution of C-3, 4 would be greater than that of C-1, since this pathway yields 2 molecules of pyruvate in which the carboxyl groups are derived from carbons three and four of glucose. If the pentose-phosphate cycle were functional, the rate of $^{14}CO_2$ evolution would be C-1 > C-2 > C-3 > C-4 > C-6. In *T. ferrooxidans*, this is clearly not the observed pattern. In the Entner-Doudoroff pathway, the glucose is split between carbon atoms three and four, with the result, however, that carbons one and four appear as the carboxyl groups of the resulting pyruvate molecules. In addition, if this pathway were the sole means of glucose degradation, it would follow that C-3 should equal C-6. If the Entner-Doudoroff pathway were operating in conjunction with any of the other pathways, C-1 would no longer equal C-4, and C-3 would not equal C-6. In these experiments CO_2 from C-6 labeled glucose was released earlier than respiratory CO_2 from C-3 labeled glucose. This was unexpected based upon established dissimilation schemes for bacteria (62). Figure 21 represents a scheme which could account for the way that glucose is dissimilated in *T. ferrooxidans*. One explanation for the high C-6 evolution is the fact that all autotrophic organisms, both photosynthetic and chemosynthetic, form fructose 1,6-diphosphate by joining one molecule of 3-phosphoglyceraldehyde and one molecule of dihydroxyacetone phosphate, essentially a reversal of the aldolase step of the Embden-Meyerhof pathway (89). In addition, autotrophs used phosphoglyceric acid kinase and phosphoglyceralde dehydrogenase in the reverse direction (89). Thus, in the case at hand, when phosphoglyceraldehyde is formed as a result of the Entner-

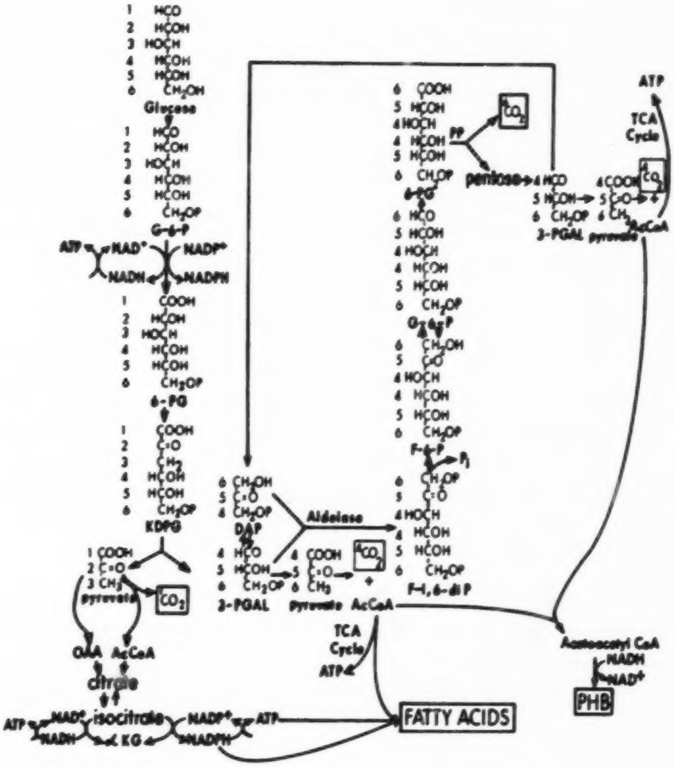

Glucose
G-6-P
ATP
NAD⁺
NADH
NADP⁺
NADPH
6-PG
KDPG
pyruvate
CO₂
OAA
AcCoA
citrate
isocitrate
αKG
Aldolase
DAP
3-PGAL
TCA Cycle
FATTY ACIDS
PP
pentose
G-6-P
F-6-P
Pᵢ
F-1,6-di P
Acetoacetyl CoA
PHB

Figure 21. Proposed pathway for glucose dissimilation in *T. ferrooxidans*. Numbers refer to position of carbon atoms in glucose. Abbreviations: G-6-P = glucose-6-phosphate; 6-PG = 6-phosphogluconate; KDPG = 2-keto-3-deoxy-6-phosphogluconate; OAA = oxaloacetate; 3-PGAL = 3-phosphoglyceraldehyde; DAP = dihydroxyacetone phosphate; F-1, 6-diP = fructose-1,6-diphosphate; F-6-P = fructose-6-phosphate; PHB = poly-β-hydroxybutyrate; PP = pentose-phosphate; Ac CoA = acetyl-CoA; αKG = α-ketoglutarate; P_i = inorganic phosphate.

Doudoroff scheme, it is able to be isomerized to dihydroxyacetone phosphate (Fig. 21). Triose phosphate isomerase has been detected in T. ferrooxidans (97). Aldolase then joins these two molecules into a molecule of fructose 1,6-diphosphate; carbon 6 (of the original glucose molecule) from 3-phosphoglyceraldehyde is now the number one carbon atom of fructose diphosphate. Fructose diphosphate can be easily converted to fructose-6-phosphate and then to glucose-6-phosphate by means of fructose diphosphatase and phosphoglucose isomerase. Phosphoglucose isomerase has been detected in extracts of T. ferrooxidans (97). Glucose-6-phosphate then recycles through the pentose phosphate pathway or through the Entner-Doudoroff pathway with the subsequent liberation of CO_2 from the original C-6 of glucose.

Glucose is dissimilated primarily (80%) through the Entner-Doudoroff scheme when T. ferrooxidans is grown on glucose. The presence of the autotrophic substrate iron (iron-glucose cells) appears to repress the Entner-Doudoroff pathway, since this pathway accounts for only 40% of the glucose dissimilated under these conditions. T. ferrooxidans is thus similar to Hydrogenomonas H-16 (92) and T. intermedius (72) in that the Entner-Doudoroff pathway is repressed by the chemolithotrophic energy source.

One of the interesting observations found was the formation of large masses of poly-ß-hydroxybutyrate granules in cells of T. ferrooxidans grown on glucose (83). The synthesis of this storage polymer requires reduced NAD^+. Reduced NAD^+ is readily available through glucose-6-phosphate dehydrogenase, and isocitrate dehydrogenase (NAD^+-linked) activity. NADH availability for possible PHB synthesis is also shown in Figure 24. $NADP^+$-linked isocitrate and glucose-6-phosphate dehydrogenases supply needed NADPH for fatty acid synthesis. The two isocitrate dehydrogenase activities are probably two distinct enzymes, since the NAD^+- and $NADP^+$-linked activities vary to different degrees depending upon how the cells are grown. The ratio of $NADP^+/NAD^+$-linked activity for glucose-6-phosphate dehydrogenase is the same regardless of how the cells are grown, thus indicating there is only one protein catalyzing these activites. This subject is discussed in the following section.

Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase catalyzes an important step in the heterotrophic metabolism of T. ferrooxidans. The enzyme uses either $NADP^+$ or NAD^+ as coenzyme based upon the constant ratio of enzyme activities with either coenzyme during each step of the enzyme purification procedure; and from results of activity staining in polyacrylamide gels. However, the $NADP^+$- and NAD^+-linked activities do respond differently to changes in pH and ATP concentration; these differences may suggest differences in the formal kinetic mechanism for these reactions (79). In addition, it was postulated that both reactions (NAD^+ and $NADP^+$) proceed by an ordered sequential mechanism, with an isomerization of free enzyme for only the NAD^+-linked reaction. It is thought that NAD^+ reacts with one form of the enzyme (E) whereas NADH, $NADP^+$ and NADPH react with another form (E') (79).

Glucose-6-phosphate dehydrogenase is identical when isolated from cells grown mixotrophically (iron-glucose grown cells) and cells grown heterotrophically (glucose-grown cells). The data showing identical electrophoretic migration patterns, similar Michaelis constants for substrates, identical $NADP^+/NAD^+$ activity ratios and similar molecular weights support this claim. In addition, extracts of autotrophically grown cells (iron-grown cells possess $NADP^+/NAD^+$ ratios identical to extracts from mixotrophically and heterotrophically grown cells. All evidence supports the fact that glucose-6-phosphate dehydrogenase is identical in properties, no matter how drastic a difference in the organism's primary energy source. Thus, there does not appear to be a different enzyme synthesized as a result of the induction by glucose, as with isocitrate dehydrogenase (72). Conversely, the low level of glucose-6-phosphate dehydrogenase found in autotrophic extracts is due to a regulation of the synthesis of the one enzyme; the organism does not have the genetic capability of synthesizing large amounts of a different glucose-6-phosphate dehydrogenase to metabolize glucose heterotrophically. It would then appear that complex regulatory processes are operating to control the synthesis of a single glucose-6-phosphate dehydrogenase. These processes are affected by the substrate milieu in which the organism is grown. In addition, the conditions of growth do not appear to induce a change in enzyme property, be it structural or catalytic.

T. ferrooxidans glucose-6-phosphate dehydrogenase is activated by Mg^{++}, and to a lesser extent by low concentrations of Mn^{++}. This property is shared by a number of bacterial glucose-6-phosphate dehydrogenases; among these sources are E. coli (77) and Pseudomonas aeruginosa. In contrast, however, glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (79) and Hydrogenomonas H-16 (78) is not activated by Mg^{++}.

the enzyme from *T. ferrooxidans*, although inhibited by NADH, is not affected in an allosteric manner, as in *E. coli*.(77) Instead, the enzyme from *T. ferrooxidans* is similar to glucose-6-phosphate dehydrogenase from the related organism, *Hydrogenomonas H-16* (78), where NADH inhibition is noncompetitive with respect to NADP$^+$. In addition, the inhibition of *T. ferrooxidans* glucose-6-phosphate dehydrogenase by ATP does not deviate from normal Michaelis-Menten kinetics as illustrated by the enzyme isolated from *Hydrogenomonas H-16* and *Ps. aeruginosa*. ATP inhibits the enzyme from *T. ferrooxidans* nearly competitively with respect to glucose-6-phosphate concentration when NADP$^+$ is used as the coenzyme; the K_i was calculated to be approximately 3 mM. However, when NAD$^+$ is the coenzyme, ATP inhibits competitively with respect to glucose-6-phosphate; in this case the K_i for ATP was determined to be 0.3 mM. There is apparently only one binding site for glucose-6-phosphate, since the slopes from the Hill plots gave values of *n* approximately equal to one. Glucose-6-phosphate dehydrogenase from another autotrophic organism, *Hydrogenomonas H-16* (78) and from the heterotroph *Ps. aeruginosa* (61), values of *n* varied from 2-3 indicating the presence of at least 2 binding sites for glucose-6-phosphate by these enzymes. The enzyme from each of these sources exhibited sigmoidal velocity versus glucose-6-phosphate concentration curves; ATP enhanced the sigmoidicity of these curves, a property not shared by the enzyme from *T. ferrooxidans*.

T. ferrooxidans glucose-6-phosphate dehydrogenase preferentially utilizes NADP$^+$; the affinity of the enzyme for NADP$^+$ is 60-80 times greater than the affinity for NAD$^+$. The glucose-6-phosphate dehydrogenase from *Hydrogenomonas H-16* (78) and *L. mesenteroides* (79) shows a 6-fold and 16-fold preference for NADP$^+$ respectively, based on Michaelis constants.

The inhibition by ATP may be important physiologically for *T. ferrooxidans*, especially with respect to the NAD$^+$-linked reaction, where the K_i is 10 times lower than that for the NADP$^+$ reaction. It has been suggested that unidentified control mechanisms linked to the respiratory system affect the entry of glucose into dissimilatory pathways (98). *T. ferrooxidans* possesses a relatively simple cytochrome chain:

$$\text{NADH} \longrightarrow \text{cyt c} \longrightarrow \text{cyt a} \longrightarrow O_2$$

The product of the oxidation of NADH through this system is ATP. Thus, the reduction of NAD^+, catalyzed by glucose-6-phosphate dehydrogenase, is needed for the subsequent generation of ATP. The ATP formed is then capable of inhibiting the action of glucose-6-phosphate dehydrogenase, by a type of product inhibition, thus regulating the flow of carbon through the Entner-Doudoroff pathway. This may be important under autotrophic conditions of growth where relatively large amounts of ATP and NADH are needed for the endergonic reduction of CO_2. In addition, the amount of glucose-6-phosphate in the cell may be a major factor in controlling its oxidation by glucose-6-phosphate dehydrogenase and the subsequent carbon flow. However, the relative concentration of glucose-6-phosphate with respect to that of ATP is also a determinant in governing the activity of glucose-6-phosphate dehydrogenase. This would appear tenable, since at high concentrations of substrate, the inhibition by ATP would be relieved.

Recently, it has been discovered that several glucose-6-phosphate dehydrogenases, including the enzyme from *T. ferrooxidans*, are regulated by acetyl coenzyme A (A. Ishaque, personal communication). It is particularly interesting that the NAD^+-reaction is inhibited to a greater extent than the $NADP^+$-linked reaction. It is conceivable that the regulation of glucose-6-phosphate dehydrogenase may be a determining factor in controlling *T. ferrooxidans*' ability for a dual existence, autotrophic or heterotrophic.

SECTION VIII

ACKNOWLEDGEMENTS

An extensive research project such as this can only be successful when full cooperation is achieved by people involved. The senior author stresses the collaborative nature of the project and thanks the many contributors involved. Recognition goes to Dr. Robie Vestal for experimental expertise and professional guidance; Dr. Gus Wang for electron microscopy and physiological techniques; Dr. Robert Tabita for vision and the pursuit of fundamentals, Dr. Carl Schnaitman for kinetic thoughts; Dr. Jerry Perry for supporting experiments, Mr. Carl Bodo, Miss Adele Howard, Mr. Robert Tuttle, and Mike Kaplan for student power and the pursuit of zealous research.

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SECTION IX

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SECTION X

LIST OF PUBLICATIONS RESULTING FROM THE GRANT

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Wang, W. S., M. S. Korczynski, and D. G. Lundgren. 1970. Cell envelope of an iron-oxidizing bacterium: Studies of lipopolysaccharide and peptidoglycan. J. Bacteriol. 104:556-565.

Howard, A. and D. G. Lundgren. 1970. Inorganic pyrophosphatase from *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). Can. J. Biochem. 48: 1302-1307.

Tabita, R., M. Kaplan and D. G. Lundgren. 1970. Microbial ecology of mine drainage. Third Symposium on Coal Mine Drainage Research. Mellon Institute, Pittsburgh, Pa. 94-113.

Tabita, R. and D. G. Lundgren. 1971. The heterotrophic metabolism of the chemolithotroph *Thiobacillus ferrooxidans*. Submitted to J. Bacteriol.

Tabita, R. and D. G. Lundgren. 1971. Utilization of glucose and the effect of organic compounds on the chemolithotroph *Thiobacillus ferrooxidans*. Submitted to J. Bacteriol.

Tabita, R. and D. G. Lundgren. 1971. Glucose-6-phosphate dehydrogenase from the chemolithotroph *Thiobacillus ferrooxidans*. Submitted to J. Bacteriol.

Vestal, J. R. and D. G. Lundgren. 1971. The sulfite oxidase of *Thiobacillus ferrooxidans* (*Ferrobacillus ferrooxidans*). Can. J. Biochem. In the press.

Lundgren, D. G., J. R. Vestal and F. R. Tabita. 1971. The microbiology of mine drainage pollution. *In* R. Mitchell (Ed.) Water Pollution Microbiology, John Wiley and Sons, Inc. New York. In the press.

SECTION XI

GLOSSARY

SO_3^{2-}	sulfite
$S_2O_3^{2-}$	thiosulfate
$S_4O_6^{2-}$	tetrathionate
DEAE-cellulose	diethylaminoethyl cellulose
Tris	tris-(hydroxymethyl)-aminomethane
Tricine	N-tris-(hydroxymethyl)-methyl glycine
EDTA	ethylenediaminetetraacetate
ΔOD or ΔA	change in optical density or absorbance
TCA cycle	tricarboxylic acid cycle
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
RuDP	ribulose diphosphate
GSH	reduced glutathione
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
cAMP	3',5'-cyclic adenosine monophosphate
APS	adenosine phosphosulfate
R_m	relative mobility
K_m	Michaelis constant
V_{max}	maximum velocity of an enzyme
K_i	enzyme inhibitor constant
9K medium	ferrous sulfate medium containing 9000 ppm Fe^{++}
9KG medium	ferrous sulfate medium with 0.5% glucose added
mole % G + C	the amount of guanine and cytosine in DNA

$S_{w,20}$	sedimentation in water at 20°C
EC number	International enzyme code number
cyt c	cytochrome *c*
cyt a	cytochrome *a*

SECTION XII

APPENDIX

Persons trained under this grant:		Present Address
Dr. Augustine Wang, Ph.D.	June 1968	Dept. of Microbiology Mississippi State Univ. State College, Mississippi
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Carl A. Bodo, Jr., Ph.D.	expected June 1972	Dept. of Biology Syracuse University Syracuse, New York
Robert C. Tuttle, B. S.	June 1971	Dept. of Biological Sciences Harvard University Cambridge, Mass.
Postdoctoral Fellow:		
Dr. J. Robie Vestal		Dept. of Biological Sciences University of Cincinnati Cincinnati, Ohio

Published abstracts of papers delivered before professional meetings.

Tabita, F. R., M. S. Silver and D. G. Lundgren. 1969. Partial purification and properties of rhodanese from *Ferrobacillus ferrooxidans*. Bacteriol. Proc. *69*:64.

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F. R. Tabita and D. G. Lundgren. 1970. Growth of iron oxidizing bacteria on different organic substrates. Central New York Branch, American Society for Microbiology (May).

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R. C. Tuttle and D. G. Lundgren. 1971. Isolation and characterization of *Thiobacillus* from alkaline mine drainage. Central New York Branch, American Society for Microbiology (March).

Titles of papers delivered at symposia at professional meetings

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Tabita, R. and D. Lundgren. 1971. Autotrophic and heterotrophic metabolism of iron-oxidizing bacteria. Symposium: Autotrophy. 71st annual meeting. American Society for Microbiology.